

# Physiology of Aspergillus niger under oxygen limitation

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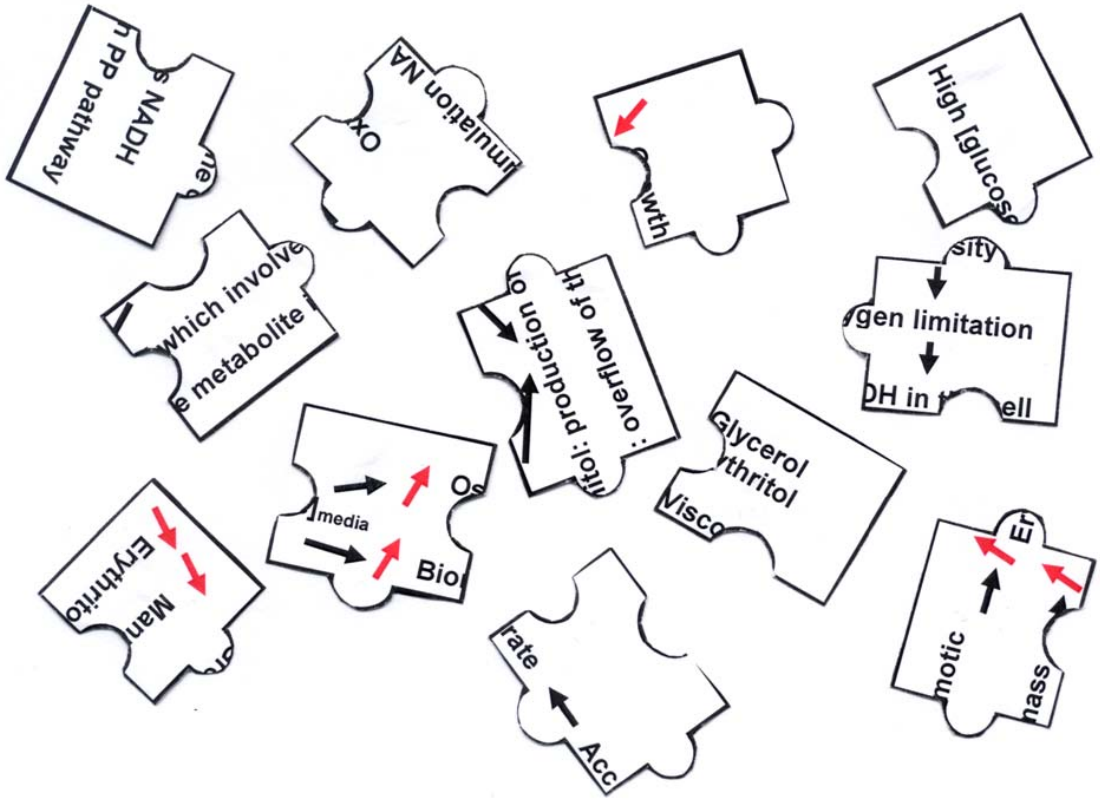


*Or how to solve a metabolic puzzle….*

BioCentrum-DTU
TECHNICAL UNIVERSITY OF DENMARK

This thesis is the result of my Ph.D. project carried out at the Center for Microbial Biotechnology (CMB), Biocentrum-DTU and at the department of Fungal Genetic at Novozymes A/S, Bagsværd.

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# SUMMARY

Filamentous fungi are extensively used in the fermentation industry for the synthesis of numerous products including primary metabolites, antibiotics, homologous and heterologous proteins. In the production of industrial enzymes, filamentous fungi, especially *Aspergillus spp.*, are among the most important cell factories, due to their efficient metabolism concerning protein secretion and to the good knowledge of fermentation technology with these organisms. However their growth as freely dispersed hyphae leads to an increase in the medium viscosity and to problem of mass transfer, especially oxygen transfer. The aim of this project was to study the physiology of *Aspergillus spp.*, particularly *Aspergillus niger*, during oxygen-limited conditions, through metabolic engineering. Experiments and strains have been designed to form a better understanding of the *A. niger* metabolism in these conditions.

The first objective was to characterize the main compounds produced during oxygen-limited conditions. Therefore, high cell density batch cultivations have been run using different carbon and nitrogen sources (Chapter 2). Metabolic quantification has showed that polyols, mainly glycerol, erythritol, xylitol and mannitol, are the main metabolites produced in oxygen limitation and represent up to 22% of the carbon consumed. However, all these polyols do not have a direct link with oxygen limitation. Indeed erythritol and xylitol seem to be involved in carbon storage compound, while glycerol, erythritol and xylitol seem to be involved in osmoregulation. The only polyol directly involved in oxygen limitation seems to be mannitol, the production of which involves the NAD-dependent mannitol-1-phosphate dehydrogenase and leads to the reoxidation of NADH.

To further understand the metabolism of *A. niger* in oxygen limitation, continuous cultures at different levels of oxygen transfer, have been run (Chapter 3). This study has shown that a decrease in oxygen availability influences rather the production of the tricarboxilic acid (TCA) cycle intermediates than the production of polyols. To further investigate these results, oxygen-limited continuous cultures have been run at different carbon source concentrations and growth rates. These experiments led to the conclusion that both carbon source concentration and growth rates are important parameters for polyol production. On one hand, the carbon source concentration affects mainly glycerol and erythritol production. Chemostats run at different osmotic pressures have confirmed the role of these polyols as osmoregulator compounds suggested by the batch cultivations, as well as the role of erythritol as carbon storage compound. On another hand, the growth rate influences particularly the production of mannitol, which may be

due to a control of the carbon catabolite repression on mannitol production. Furthermore, the study of the physiology of *A. niger* under extreme oxygen-limited conditions has confirmed that mannitol production is the main cellular answer helping the reoxidation of NADH when the final electron transporter of the respiratory chain, $O_2$, is limiting.

In a third part of this Ph.D., the gene encoding for the NAD-dependent mannitol-1-phosphate dehydrogenase has been deleted as well as the gene encoding for the NAD-dependent glycerol-3-phosphate dehydrogenase leading to the production of glycerol (Chapter 4). Both simple and double deletion mutants have been constructed. The objective of this study was to check the role of these dehydrogenases, involved in the production of mannitol and glycerol, two important polyols which can represent up to 19% of the carbon consumed during high density batch cultivations. This study revealed an important role of these two enzymes during non-limited growth conditions, presumably due to their involvement in the synthesis of cell wall components. Moreover, this study has demonstrated that the presence of the NAD-dependent mannitol-1-phosphate dehydrogenase activity is important but not vital during oxygen limitation, while, in this condition, the role of the NAD-dependent glycerol-3-phosphate dehydrogenase is limited.

Finally, in order to improve the availability of oxygen in high cell density batch cultivation, the gene *vgb*, encoding for the Vitreoscilla hemoglobin protein has been expressed in *A. niger* (Chapter 5). In oxygen-limited conditions, the increase of biomass production rate and glucose consumption rate, as well as the decrease of the yield of mannitol and ethanol, indicate a release of oxygen stress for the strain expressing the hemoglobin gene. One of the possible roles of hemoglobin is temporary storage of oxygen. Indeed filamentous fungi broths are highly viscous and lead to non homogeneous solutions where the repartition of dissolved oxygen varies in the fermentor. The cells experience successively period of hypoxia and period of anoxia. The hemoglobin could thus capture oxygen in the aerated zone and release it intracellularly in the non-aerated zone, attenuating the stress causing by the heterogeneity of the oxygen repartition.

# DANISH SUMMARY

Skimmelsvampe anvendes i fermenteringsindustrien til at producere en række forskellige produkter, såsom organiske syrer, antibiotika, enzymer og andre proteiner. I forbindelse med produktion af industrielle enzymer anvendes der ofte arter af *Aspergillus*, idet disse organismer er velkendte for deres gode protein sekretion. Et betydeligt problem i forbindelse med fermentering med disse skimmelsvampe er dog at de danner et forgrenet netværk af celler som fører til en høj viskositet af fermenteringsmediet. Dette skaber problemer med ilttilførslen, som direkte ændrer stofskiftet i skimmelsvampene til dannelse af biprodukter, som f.eks. glycerol, mannitol og xylitol. Formålet med dette projekt var at karakterisere stofskiftet af *Aspergillus niger* under iltbegrænsning for at identificere mekanismerne bag biprodukt dannelsen og efterfølgende reducere denne ved hjælp af metabolic engineering.

Til fysiologisk karakterisering af *A. niger* blev der anvendt både batch fermenteringer og kemostater. Batch fermenteringer blev gennemført med en meget høj sukkerkoncentration, så der i den sidste del af fermenteringen opstod iltbegrænsning. Igennem disse batch fermenteringer blev dannelsen af polyoler såsom glycerol, erythritol, xylitol og mannitol målt, og det blev fundet at op til 22% af det forbrugte sukker blev omdannet til disse biprodukter. Dannelse af polyoler er hidtil blevet forklaret med, at der er en begrænsning i oxidationen af NADH under iltfattige forhold. Resultaterne viste dog, at kun dannelsen af mannitol kan forklares ved denne mekanisme, og at dannelsen af glycerol, erythritol og xylitol snarere synes at være koblet til osmoregulering. Yderligere fermenteringsforsøg under iltbegrænsing i kemostater bekræftede dette, og viste specielt at mannitol er det primære biprodukt, der dannes under ekstreme iltbegrænsninger.

I den sidste del af PhD projektet blev de gener, der koder for den NAD-afhængige mannitol-1-phosphate dehydrogenase og for den NAD-afhængige glycerol-3-phosphate dehydrogenase, deleteret. Både enkelt og dobbelt deletions mutanter blev konstrueret. Formålet med dette studium var yderligere at undersøge rollen af disse enzymer i osmo- og redoxregulering i *A. niger*, og den hypotese, der blev opstillet fra de tidligere forsøg, blev bekræftet. Endvidere blev det påvist, at det var muligt at reducere biprodukt dannelsen betydeligt ved deletion af disse gener.

Som et yderligere forsøg på at minimere dannelsen af biprodukter, blev der udtrykt et gen, *vgb*, der koder for en bakteriel hemoglobin. Udtrykkelsen af denne hemoglobin vi-

ste sig at have en positiv indflydelse på biprodukt dannelsen da der blev dannet betydeligt lavere niveauer af mannitol under iltbegrænsning.

PhD projektet har således demonstreret, hvordan det er muligt på basis af en grundlæggende karakterisering af en mikroorganisme at designe rationelle strategier til gensplejsning med henblik på at opnå forbedrede produktionsegenskaber.

## CONTENTS

# I. Bibliography

## *1.* Classification and characteristics of *Aspergillus spp.*

*Aspergillus spp.* belong to the superkingdom of Eukaryota (Figure 1). Their cells are compartmentalized in nuclei and organelles and the main part of the genetic information is carried by chromosomes localised in the nucleus. In 1969, Robert Whittaker proposed a division of the superkingdom of Eukaryota into 4 kingdoms based on structural and nutritional criteria. These kingdoms are: the protozoa and the unicellular algae (kingdom of Protoctista or Protista), the photosynthetic plants (Kingdom of Plantae), the absorptive fungi (Kingdom of Fungi), and the ingestive animals (Kingdom of Animalia).

**Eukaryota** (superkingdom)
**Fungi** (kingdom)
**Ascomyceta** (phylum)
**Pezizomycotina** (subphylum)
**Eurotiomycetes** (class)
**Eurotiales** (order)
**Trichocomaceae** (family)
**Mitosporic trichocomaceae**
*Aspergillus niger*
*Aspergillus oryzae*
*Penicillium spp.*
**Emericella**
*Emericella nidulans*

Figure 1 - Taxonomic classification of *Aspergillus spp.* According to NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Taxonomy)

*Aspergillus spp.* belong to the kingdom of fungi, which is characterized by a development from spores, as well as an absorption of nutrient into the cell (Carlile and Watkinson, 1994). This kingdom shares some similarities with the Animalia kingdom such as chitinuous structures, storage of glycogen and UGA coding for tryptophane, as well as with the Plantae kingdom such as the presence of cell wall surrounding the cell. The number of fungal species is estimated to be 1.5 millions (Hawksworth, 2001), which ranks this kingdom in second place behind animals. Fungi are chemotrophic and het-

erotrophic organisms. They have an important role in the nature as saprophyte, parasite or symbiont organisms. First as saprophytes, fungi, along with bacteria, are the main decomposers of dead organic matters. As pathogens, they can cause serious diseases in many organisms, particularly in immuno-compromised organisms. A typical example is *Aspergillus fumigatus,* which is a pathogen of immuno-compromised plant and animal cells. Finally, as symbiont, fungi have important symbiotic relationships and are engaged in mycorrhizal relationships with over 90% of plant species. The ecological niches of fungi are therefore diverse, which is notably due to their capacity of adaptability to different environments (Schuster et al., 2002; Tekaia and Latge, 2005) and to their ability of degrading a wide range of organic compounds. These organic compounds are first hydrolyzed externally and then absorbed into the cell.

The fungal kingdom is divided into phyla, based traditionally on their reproduction properties. In this traditional classification, most of the *Aspergilli* were regrouped under the phylum of asexual fungi called Deuteromycetes. However, other *Aspergillus spp.*, like *Aspergillus nidulans*, have a sexual reproductive structure and were put under the phylum of Ascomycetes and under the genus of Emericella. More recently, the use of DNA sequences analysis has allowed regrouping all *Aspergillus spp.* to their closest relative, under the phylum of Ascomycetes, in the subphylum including the major lines of filamentous fungi, Pezizomycotina, and in the family of Trichocomacae (Bills et al., 2005; Sidow and Thomas, 1994).

In this family, *Aspergillus* was first described in 1729 by the Italian priest and biologist Pietro Antonio Micheli in his book *'Nova plantarum genera juxta Tournafortii methodum disposita'*. The shape of the conidiophores of this organism, similar to an aspergillum, an instrument used to disperse holy water in catholic churches, gave its name (Figure 2). *Aspergillus niger* is one of the *Aspergillus spp.* and got its name from its black conidiospores (Schuster et al., 2002).

Figure 2 - Comparison between an aspergillum (left) and a conidiophore of *Aspergillus spp.* (right)

## 2. Importance of *Aspergillus spp.* in biotechnology

The natural characteristics of *Aspergillus spp.*, described previously, make them very attractive for diverse industrial purposes. Particularly, their ability for degrading externally a wide range of organic compounds is nowadays exploited and *Aspergilli* are widely used as cell factories for the production of enzymes (Burlingame and Verdoes, 2006). The use of *Aspergillus* for industrial production started some centuries ago in Asia with the production of "sake", rice wine (produced by *A. oryzae*), "shoyu" and "tamari", soy sauces (produced by *A. oryzae*, *A. tamarii* and *A. sojae*) and "miso", soybean paste (produced by *A. oryzae*) (Carlile and Watkinson, 1994; Machida, 2002; Steinkraus, 1983). However, the first deliberate biotechnological process using fungi is registered to be in the late XIX$^{th}$ century with the existence of a patent handling on the production of alpha amylase by *A. oryzae* (Bodie et al., 1994). Since then the market of enzymes has expanded, first to baking and brewing industry (Barbesgaard et al., 1992), and later to textile, detergent, paper, leather and food industries. Nowadays, this market is in continuous expansion due to the development of a number of new products, to the improvement of the efficiency of several existing products and to the expansion of their application field, e.g. in the bioethanol industry. In 1998, the enzymes market reached US$ 1.6 billions divided into the following application areas: 45% food, 34% detergents, 11% textiles, 3% leather and 1.2% pulp and paper (Demain et al., 2005). According to the report from Business Communications Company, Inc. (RC-147U Enzymes for Industrial Applications), the global market for industrial enzymes was estimated at $2 billion in 2004 and should increase up to 2.4 billion in 2009. Numbers of processes have been Generally Recognized As Safe by the US Food Administration and today, *Aspergillus spp.*, which are the most important fungi for commercial enzyme production, are used both for the production of homologous and heterologous enzymes (tables 1 and 2). Their advantages compared to the traditional host *Escherichia coli* or *Saccharomyces cerevisiae* comprise their efficiency to secrete high amounts of proteins (Wösten et al., 1991) and the existence of intron splicing and post translational mechanisms, such as glycosylation, allowing the correct processing of proteins originally produced in higher eukaryotic cells. The principal strains used nowadays for heterologous protein expression are *A. niger*, *A. awamori* and *A. oryzae* due to the high level of recombinant proteins obtained with these strains and to the accumulated knowledge on their fermentations. However, there are several factors limiting the production yield of heterologous enzymes in filamentous fungi, such as transcription and translation control, mRNA stability, secretion and extracellular degradation, particularly proteolityc degradation. These limiting steps have been studied during the past 10 years in order to

increase the yield of heterologous protein expression (Gouka et al., 1997; Zhang et al., 2005).

| Enzyme | Industry | Source | Reference |
|---|---|---|---|
| alpha amylase glucoamylase | Food processing, baking and miling Starch degradation | *A. oryzae* *A. niger* | (Bodie et al., 1994; Carlsen and Nielsen, 2001; Schuster et al., 2002) |
| Protease | Baking, cheese detergent, food and beverage protein hydrolysates | Various | (Bodie et al., 1994) |
| Lactase | Milk | *A. oryzae, A. niger* | (Bodie et al., 1994) |
| Pectinase | Fruit juices and wine (clarification) Coffee bean fermentation | *A. niger* | (Bodie et al., 1994; Schuster et al., 2002) |
| Glucose oxidase Catalase | Fruit juices, soft drink, flavors gluconic acid production | *A. niger* | (Bodie et al., 1994; Schuster et al., 2002) |
| Hemicellulase | Baking | *A. niger* | (Schuster et al., 2002) |
| Xylanase | Brewery, Baking, paper and pulp | *A. niger, A. phoenicis* | (Bodie et al., 1994) |

Table 1 - Examples of homologous enzymes produced by *Aspergillus spp.*

| Enzyme | Industry | Source | Reference |
|---|---|---|---|
| Aspartyl protease, chymosin | Cheese | Various | (Christensen et al., 1988; Demain et al., 2005) |
| Lipase | Detergent | *A. oryzae* | (Boel and Huge-Jensen, 1989; Demain et al., 2005) |
| Human Interleukins | Pharmaceutical | *A. niger* | (Broekhuijsen et al., 1993; Demain et al., 2005) |
| Lysozyme | Pharmaceutical, dairy, cheese, wine | *A. niger* | (Demain et al., 2005) |
| Human lactoferrin | Food, pharmaceutical | *A. oryzae* | (Ward et al., 1995) |

Table 2 - Examples of heterologous enzymes produced by *Aspergillus spp.* For a detailed list of heterologous fungal enzymes see Verdoes et al. (1995), of heterologous non fungal enzymes see Gouka et al. (1997).

*Aspergillus spp.* have also been extensively used for the production of organic acids particularly for the production of citric acid and gluconic acid using *A. niger*, and for the production of itaconic acid using *A. terreus* (Ruijter et al., 2002). The use of filamentous fungi for citric acid production started in 1917, when Currie isolated an *A. niger* strain capable of producing large amount of citric acid. The first large scale production was started in 1923 by the company Chas Pfizer and Co Inc. Since then, the production of citrate has continuously increased. Nowadays citric acid is mostly used in food, soft drink and pharmaceutical preparations and the global production was estimated at 900 000 tons in 2000 (Ruijter et al., 2002). However, although high yields are obtained in industrial process, the metabolism leading to the accumulation of citric acid is not completely understood (Karaffa and Kubicek, 2003). High citric acid productivity has

also been obtained by improving the fermentation process. The first production plants used solid phase fermentation processes. In 1932, the possibility of cultivating *Aspergillus* in submerged fermentation was demonstrated (Schuster et al., 2002). Such fermentation results in a less labor intensive process than a surface fermentation and in high production rate (Bodie et al., 1994). However, it is only in the second half of the XX$^{th}$ century that the fungal fermentation process gradually changed from surface culture to stirred tank fermentation.

Lately, *Aspergillus spp.* have been used for the production of secondary metabolites. Indeed, filamentous fungi can synthesize a wide range of complex molecules using established metabolic pathways. These metabolites may be biologically active and can be utilized in pharmaceutic, like the anticholoesterol agent lovastatin produced by *A. terreus* (Manzoni and Rollini, 2002). However, many secondary metabolites can also be harmful such as aflatoxin, a powerful carcinogenic substance produced by *Aspergillus flavus* and *Aspergillus parasiticus*.

*Aspergillus spp.* can also be used for the synthesis of other products like thaumatine, a sweetness protein originally from the plant *Thaumatococcus danielli*, which can be produced by *A. niger var awamori* (Demain et al., 2005).

## 3. Growth and morphology

*Aspergillus spp.* grows by apical elongation forming a filament named hyphae (Wessels, 1993). This elongation involves the extension of the cytoplasmic membrane and of the cell wall, which is done by the discharge of vesicles containing lytic and biosynthetic enzymes at the apex (Carlile and Watkinson, 1994). The main component of the cell wall is chitin. As the hyphae grows, the cell wall becomes more rigid by cross linking of chitin, glucan components as well as by the addition of further materials. The elongation of the hyphae and the polarization of growth are mediated by a highly dynamic cellular structure named Spitzenkörper or apical body, which is located at or near the tip (Virag and Harris, 2006). Along the hyphal growth, a compartmentalization occurs via perforated membranes named septa, which allows exchange between the compartments. In the cytoplasm, close to the pore of each septum, there are often one or more appreciatively spherical structures named Woronin bodies. A Woronin body may move to block the pore if the adjacent cell is damaged (Tenney et al., 2000). Each cell or compartment may have several nuclei. These compartments are named apical when located at the tip of the hyphae or subapical when located elsewhere. Another compartment, named hyphal compartment, has been described by Nielsen (1993) as the older vacuolated parts of the hyphal element. Subapical cells can branch forming a new tip and a new growing hyphae. This branching system allows an occupation of the three dimen-

sional space, which increases the probability of finding new nutrients. *Aspergillus spp.* can also produce some specialized aerial hyphae named conidiophores and carrying conidiospores, which release spores and allow the propagation of the specie by asexual reproduction. A sexual reproduction has been observed in some *Aspergillus spp.,* like *A. nidulans*, also called *Emericella nidulans*. This sexual process involves the production of haploid ascospores through the division of diploids heterokaryons, which are then released in the environment (Figure 3).

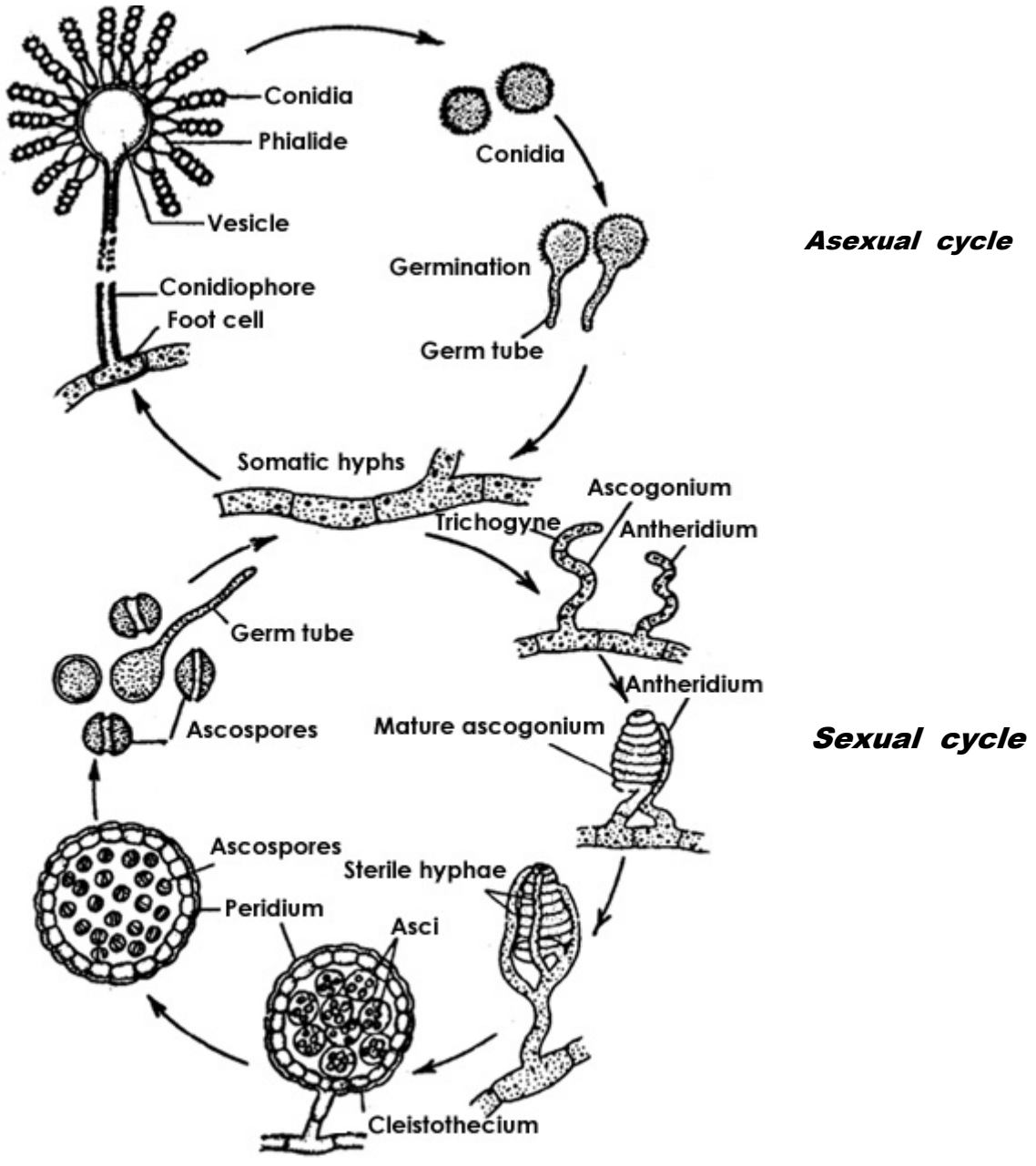


Figure 3 - The life cycle of *A. nidulans* from the Food and Agriculture Organisation of the United Nations:
http://www.fao.org/inpho/content/compend/text/ch31/ch31_04.htm

## 4. Morphology and mass transfer

In submerged cultivations, depending on the conditions, the morphology of filamentous fungi lies between a compact form, often spherical, named pellet and a free dispersed form named filamentous (Figure 4). In between these two extreme morphologies, filamentous fungi can also growth as loose aggregates named clumps. The parameters determining the type of growth are: the inoculum level (Papagianni and Mattey, 2006), the pH (Grimm et al., 2005), the concentration of trace elements (Majolli and Aguirre, 1999; Pera and Callieri, 1997), the agitation (Gomez et al., 1988; Grimm et al., 2005; Papagianni et al., 1998) and the aeration (Cui et al., 1998; Grimm et al., 2005). Moreover the hyphal morphology itself can vary from linear filament to highly branched structure. The branching intensity decreases in response to low concentration of oxygen (Wongwicharn et al., 1999), at low agitation (Amanullah et al., 2002) and at low growth rate (Wiebe and Trinci, 1991).

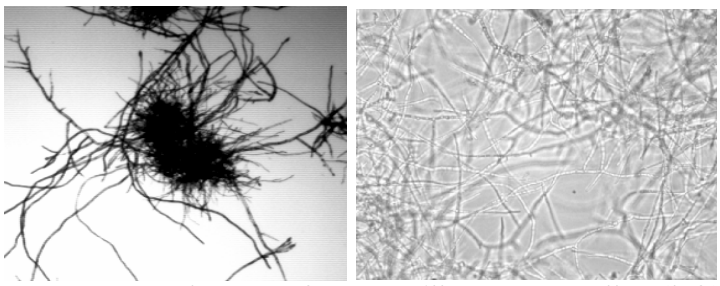

Figure 4 - The two extreme shapes of *Aspergillus spp.*: pellet (left) and filamentous (right)

The pellet form is mainly used for the production of citric acid (Bodie et al., 1994). This shape is initiated by an agglomeration of spores or hyphae (Dynesen and Nielsen, 2003). One of the disadvantages of this growth is the difficulty to control the size of the pellet. As the pellet size increases, the diffusion of substrate within the pellet decreases (Kobayashi et al., 1973), which reduces the productivity of many processes (Abarca et al., 2004; Michel, Jr. et al., 1992). The filament form is thus often used for the production of homologous and heterologous proteins or secondary metabolites (Gyamerah et al., 2002; Paul et al., 1994; Shiba et al., 2001). However, the growth as filament and the entanglement of hyphae result in a formation of network (Figure 4). As the biomass concentration increases, the mycelial branched network increases the viscosity of the medium, leading to a non Newtonian fluid behavior, having relatively low viscosity in region of high shear and high viscosity as the shear decreases. In stirred tank, the most important type of bioreactor used in bioindustry, such fluid behavior leads to a non-homogeneous broth, well mixed near the impellers, where the shear is high, but with limiting mixing elsewhere (Reuss et al., 1982; Li et al., 2002). At high biomass concen-

tration, the presence of slow moving or stagnant broth areas are observed near the fermentor wall (Wernau, 1985). In these conditions, the entrance of titrant controlling the pH and the entrance of the pH probe must be carefully positioned, in order to avoid large pH deviations. The extracellular pH has indeed a lot of importance in *Aspergillus* fermentation. First, it influences the production of acids: while citric acid can be produced at low pH, production of oxalic and gluconic acids are optimal at pH 5-6 (Ruijter et al., 2002). Secondly, pH has been reported to have an influence on the morphology of filamentous fungi: Pirt and Callow (1959) and Miles and Trinci (1983) showed a relation between the extracellular pH and the branching intensity as well as the hyphal diameter of *Penicillium chrysogenum*, which is another filamentous fungi from the same subfamily as *Aspergillus spp.* However, such a correlation has been rejected by Van Suijdam and Metz (1981). This may be due to the difference in the mode of cultivation: chemostat for Pirt and Callow and Miles and Trinci, and batch cultivations for van Suijdam and Metz (1981).

Furthermore, mixing problems also lead to heat transfer problems. Heat transfer is usually achieved by intern loops or by an external jacket. The existence of slow moving or stagnant areas near the fermentor wall is a problem for temperature control, especially when the cooling system is located nearby the reactor wall.

Bad mixing also affects the repartition of products and nutrients, in particular during continuous cultures and fed batch cultivations. Here also the position of the feed port has to be considered carefully in order to limit the gradient of substrate. Mixing problems affect as well the transfer of gas, especially oxygen, which is problematic for aerobic fermentation processes. The maximal dissolved oxygen concentration in a liquid is limited to 0.26 mmol $O_2$/L at 25°C. This maximal concentration is affected by the existence of stagnant films causing resistance to the transfer at the gas/liquid interface and at the liquid/cell interface (Figure 5). The most important resistance in Newtonian media is generally considered to be the liquid film resistance around the bubble (Kobayashi et al., 1973). However in non-newtonian cultures such as filamentous fungi broths, the main resistance has been shown to be downstream of the air/liquid interface (Li et al., 2002). The thickness of these films depends on the degree of turbulence and on the physical properties of the medium: it increases with viscosity (Barberel and Walker, 2000), causing a decrease of oxygen transfer. Moreover, the mass transfer is proportional to the kla coefficient, where “kl” is the mass transfer coefficient and “a” is the gas/liquid interfacial area per unit liquid volume. Thus the mass transfer from the gas phase to the liquid phase is also decreased by the formation of large coalescent air bubbles, which are formed due to bad mixing and high viscosity and which reduce the exchange surface (Figure 6). The repartition of air bubbles is affected as well by the bad mixing, which means that oxygen transfer is mainly taking place near the impeller re-

gion, leading to an heterogeneity of dissolved oxygen levels in the fermentor (Manfredini et al., 1983). Furthermore, the high viscosity and the low mixing also limit the transfer of oxygen within the liquid phase.

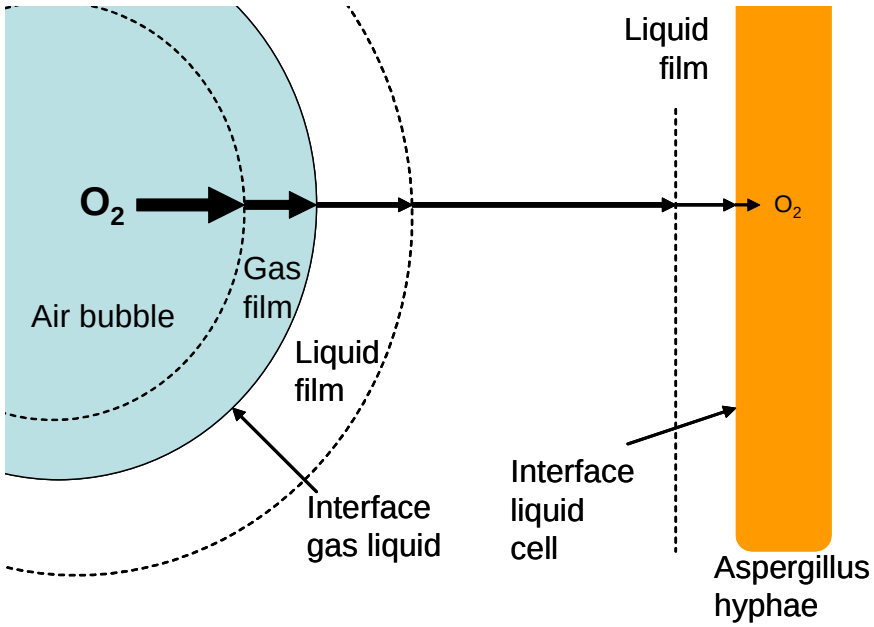


Figure 5 - Oxygen transfer from the air phase to *Aspergillus* cell. The thickness of the arrow indicates the concentration of the oxygen and shows the resistance encountered during the transfer.

Different parameters can be adjusted for better mixing and transfer such as the number, shape and size of the impellers (reviewed by Nordkvist, 2005), as well as the position of impellers and the impellers speed (Loucaides and McManamey, 1973). However, increasing the agitation is costly for industrial fermentation and doesn't always lead to an increase in productivity. Indeed high agitation level affects the morphology by fragmenting the hyphae and may lead to a decrease of productivity (Makagiansar et al., 1993; Smith and Lilly, 1990). Moreover, in 2002, Li et al. pointed out that in non-newtonian broth, better mixing does not always lead to better oxygen transfer and that oxygen transfer depends not only on the agitation power but also on how this power is applied to the culture. Another solution to improve mass transfer is the use of pulsed feeding. Most fungal industrial bioprocesses are carried out using fed batch cultivations and studies have shown the advantages of pulsed feeding leading to smaller fungal elements, to a lower medium viscosity and thereby to a better oxygen transfer (Bhargava et al., 2003b; Bhargava et al., 2003a). Another way to improve mixing and transfer is the use of a rotary jet instead of impellers to mix the reactor (Nordkvist, 2005). In such a system, the broth is drawn from the bottom of the fermentor, circulated by a pump and reinjected in the fermentor by a rotary jet located in the fermentation medium. Liquid feed, titrants and gas can be added in the loop, thus limiting the formation of gradients.

Heat exchange can also be performed in the loop via a heat exchanger. If this system presents many advantages (Nordkvist et al., 2003; Nordkvist, 2005), it may, however, be difficult to apply to filamentous fungi fermentation as the shear stress in the loop and at the exit of the rotary jet may damage the morphology of the fungi and thereby may affect the productivity.

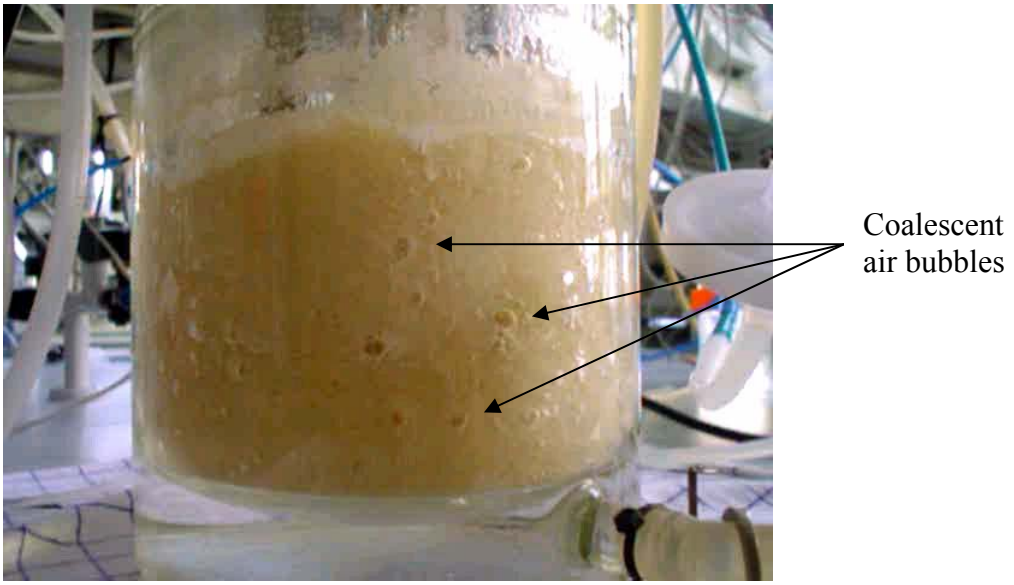


Figure 6 - Picture of a highly viscous *Aspergillus niger* broth.

## 5. Influence of oxygen on the morphology and physiology of *Aspergillus spp.*

Oxygen transfer has been largely studied to improve processes since oxygen plays an important role on the cell metabolism (Chapter 3) and affects both the morphology and physiology of the microorganisms.

### a. Morphology

Wongwicharn et al. (1999) and Rahardjo et al. (2005) have shown that a decrease of Dissolved Oxygen Tension (DOT) down to 0% reduces the branching intensity of *A. oryzae*, increases the number of vacuoles in the hyphae and decreases the percentage of active length. The active length corresponds to the length between the apex and the beginning of the vacuolated zone. Katz et al. (1972) suggested that the reduced formation of branches in a medium unfavourable for growth results from a natural selection. Indeed, this phenomenon enables the fungi to stretch for areas richer in nutrient, with the formation of a minimum amount of biomass. However, in submerged fermentation this leads to an increase of medium viscosity, which in turn decreases the oxygen

transfer. On the other hand, Carter and Bull (1971) have shown that a decrease of oxygen availability up to 1 mmHg (DOT inferior to 1%) does not affect the branching intensity of *A. nidulans*. The morphology is however affected below 18mmHg (DOT around 10%) with the apparition of large swollen cells. At even lower oxygen tension, free conidia were observed in the medium. No effect of oxygen on the branching intensity has been noticed in *A. niger* within a range of DOT from 40 to 7% (Olsvik et al., 1993) and in *P. chrysogenum* from 12 to 300 mmHg (7.5 to 188% DOT) (van Suijdam and Metz, 1981). Moreover, in pellet cultivation of *A. awamori*, dissolved oxygen tension from 5 to 330% have no influence on the pellet size, the hairy length of pellets and the free filamentous mycelial fraction. Below 5%, the pellet becomes weak and fluffy, while above 330% of air saturation the pellets are denser and the free mycelial fraction quasi inexistent (Cui et al., 1998). In conclusion, the effect of oxygen on the morphology of filamentous fungi seems to be strain dependent and is mainly observed at very low or very high DOT. However, it has been shown that under a DOT of 10% the viscosity of an *A. niger* broth increases proportionally with the decrease of oxygen tension even though the morphology of the mycelium is not affected (Olsvik et al., 1993; Olsvik and Kristiansen, 1992). This could be the result of a change in hyphal flexibility, as suggested by Zetelaki (1970). Another hypothesis has been formulated by Olsvik et al. (1993) based on image analysis: the authors concluded that in most cases, the so-called freely dispersed hyphae broth, observed visually, is in fact a non homogeneous suspension of aggregated filaments forming clumps. In this study, no influence of DOT on the macromorphology of the filaments was observed, but the authors measured an increase of the roughness of the clumps (quantification of the clump shape) as the DOT decreased. This might suggest that the morphology of the clumps more than the filaments itself exerts an influence on the broth viscosity. The importance of clump formation in mycelial broth rheological properties has also been demonstrated in *P. chrysogenum* fermentations (Packer and Thomas, 1990).

### b. Physiology

A decrease of oxygen availability affects the growth rate (Khan and Ghose, 1973; Rahardjo et al., 2005), the specific production rate of primary metabolites like citric acid (Khan and Ghose, 1973; Kubicek et al., 1980) and itaconic acid (Gyamerah, 1995), the specific production rate of secondary metabolites, like penicillin (Vardar and Lilly, 1982) and lovastatin (Casas Lopez et al., 2005), as well as the production of enzymes like alpha-amylase (Amanullah et al., 2002; Rahardjo et al., 2005) and glucoamylase (Wongwicharn et al., 1999). At low oxygen availability, the production of these metabolites stops and is often impaired irreversibly. This is due to the important roles played by oxygen at different levels of the cell metabolism. Its principal role is to be the final

electron acceptor in the mitochondria respiratory chain. This function is essential for aerobic microorganisms, as it allows the reoxidation of NADH and $FADH_2$ as well as the production of ATP. The consequences of a lack of oxygen are summarized in figure 7 and further discussed in chapter 3. However, the regulation mechanisms induced by low oxygen levels in *Aspergillus spp* are far from understood. More studies have been carried out in *S. cerevisiae,* which underline the presence of a hypoxic signal regulating the expression of specific genes, like, for example, the genes encoding for the cytochromes of the respiratory chain (Burke et al., 1997; Castello et al., 2006; Kwast et al., 1999). However, it is not clear yet how the oxygen concentration is sensed and how this signal is transmitted.

Oxygen also plays a role as nutrient. Indeed oxygen can be incorporated into unsaturated fatty acids and sterols. This incorporation of oxygen has been principally studied in *S. cerevisiae*. Concerning the production of ergosterol, 12 molecules of oxygen are required for the conversion of squalene to ergosterol, while for the biosynthesis of unsaturated fatty acids, one molecule of oxygen is required for the formation of each double bond (Burlingame and Verdoes, 2006). Data on these pathways in *Aspergillus spp.* are scarce. However, equivalents to the genes *ERG1* and *ERG3* from *S. cerevisiae,* coding for enzymes requiring oxygen, have been localised in *A. fumigatus* and *A. nidulans* (Metz et al., 2006; Tekaia and Latge, 2005). Finally, another role of oxygen is its involvement in the synthesis of secondary metabolites, like penicillin (Vardar and Lilly, 1982) and lovastatin (Casas Lopez et al., 2005).

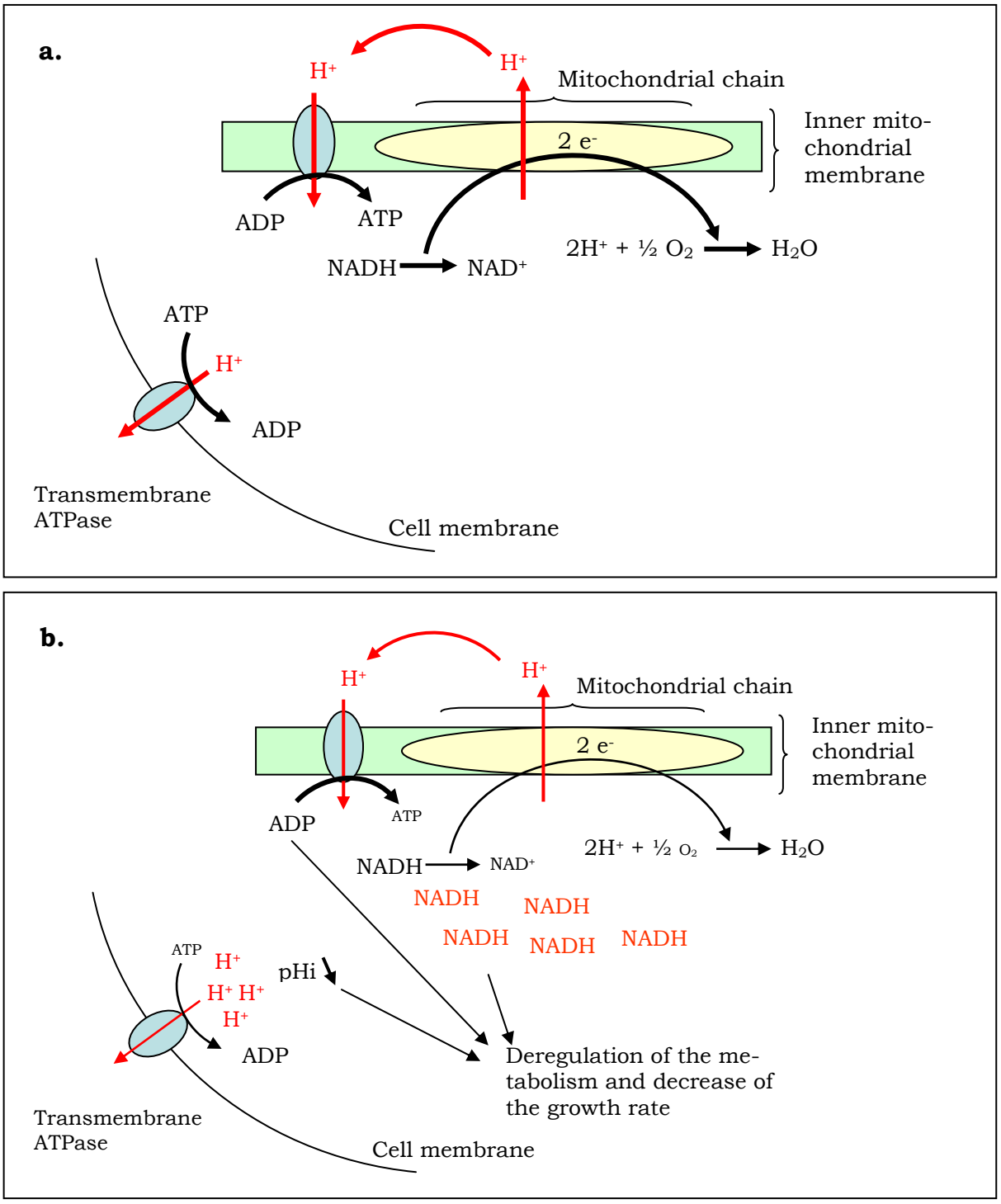


Figure 7 - Effect of low oxygen availability on the metabolism of *A. niger*. **a**. represents the cell state in fully aerobic conditions. **b**. represents the cell state in oxygen limitation conditions: low oxygen levels reduce the activity of the mitochondrial chain. The reoxydation of NADH decreases, which may lead to an intracellular accumulation of NADH. In parallel, the ATP production is likely to decrease, which could affect the transmembranaire ATPases. This could lead to an intracellular accumulation of protons and to a decrease in the intracellular pH ($pH_i$). The unfavorable energetic state added to a decrease in the $pH_i$ is likely to lead to a deregulation of the carbon flux and to a decrease in the growth rate (cf chapter 3).

These different roles of oxygen imply that low oxygen levels affect the cell metabolism in many ways. Besides decreasing the growth rate and the production of the metabolites mentionned above, low oxygen levels also activate specific pathways, in order to cope with the high catabolic reduction of charge defined as the ratio NADH on NAD + NADH. In *Aspergillus spp.,* low oxygen levels activate ethanol pathway, which allows the regeneration of NAD (Bradshaw et al., 2001; Felenbok et al., 2001). The production of large amount of polyols has also been observed in oxygen-limited industrial fermentation of *A. niger* and this matter is further discussed in the next chapter. The decrease in growth rate, as well as the decrease of production of compounds of interest like secondary metabolites and enzymes, and the loss of carbon due to the production of ethanol, acetate and polyols, imply that periods of oxygen limitation should generally be avoided in industrial processes. However, oxygen limitation may be advantageous in some processes, for example, to avoid the production of organic acids like oxalate, which create difficulties for the downstream processing.

## 6. Introduction to the thesis

Before starting with the experimental part of this thesis, one should first notice that it is difficult to differentiate the effects of oxygen from the ones exerted by carbon dioxide, as low oxygen levels generally imply low carbon dioxide levels. The carbon dioxide effects have been reviewed by McIntyre and McNeil (1998). Low levels of dissolved carbon dioxide may affect the growth, but in this work, oxygen effects have been considered as preponderant compared to the ones exercised by carbon dioxyde.

Furthermore, during this study *A. niger* has been grown as filaments. As explained earlier, this kind of growth may cause different problems due to the high viscosity of the medium, besides low oxygen transfer. Typical problems are the apparition of gradients of pH and temperature. Therefore, during the high density batch cultivations run in this project, titrant ports and pH probes have been localised as close as possible to the impellers, while temperature control was maintained using an internal loop.

# II. Polyol synthesis in *Aspergillus niger*: influence of oxygen availability, carbon and nitrogen sources on the metabolism.

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## 1. Abstract

Polyol production has been studied in *Aspergillus niger* under different conditions. Fermentations have been run using high concentration of glucose or xylose as carbon source and ammonium or nitrate as nitrogen source. The growth of biomass, as freely dispersed hyphae, led to an increase of medium viscosity and hereby a decrease in mass transfer, especially oxygen transfer. The consequence was a decrease in DOT and the occurrence of a switch between fully aerobic conditions and oxygen-limited conditions. Metabolite quantification showed that polyols were the main metabolic products formed and represented up to 22% of the carbon consumed in oxygen-limited conditions. The polyol concentration and the polyol pattern depended strongly on the environmental conditions. This is due to a complex regulation of polyol production and to the fact that each polyol can fulfill different functions. In this study, erythritol, xylitol and arabitol were produced as carbon storage compounds when the flux through the pentose phosphate (PP) pathway exceeded the need in ribulose-5-phosphate for the biomass synthesis. Glycerol, erythritol and xylitol seem to be involved in osmoregulation. Mannitol was produced when the catabolic reduction of charge was high. Its production involves the enzyme NAD-dependent mannitol-1-phosphate dehydrogenase and seems to be the main cytosolic route for the NADH reoxidation during oxygen limitation.

## 2. Introduction

Filamentous fungi are extensively used in the industry for the production of numerous products including primary metabolites, heterologous proteins and industrial enzymes. Cultivation of filamentous fungi as freely dispersed hyphae leads to an increase in the medium viscosity and to problems in mass transfer, especially oxygen transfer. Low oxygen transfer may lead to a period of oxygen limitation, which causes problems in industry, often due to the production of large amounts of polyols. These polyols are mainly mannitol, glycerol, erythritol and arabitol, whereas other polyols like xylitol, ribitol, D-threitol and dulcitol are produced at specific environmental conditions (Blumenthal, 1976; Corina and Munday, 1971; Lewis and Smith, 1967).

The pathways for polyol biosynthesis and catabolism can be different from one *Aspergillus sp.* to another. These pathways have mainly been studied in *A. nidulans* and *A. niger*. Figure 1 shows an overview of the pathways identified in *A. niger* (David et al., 2003; Ruijter et al., 2003).

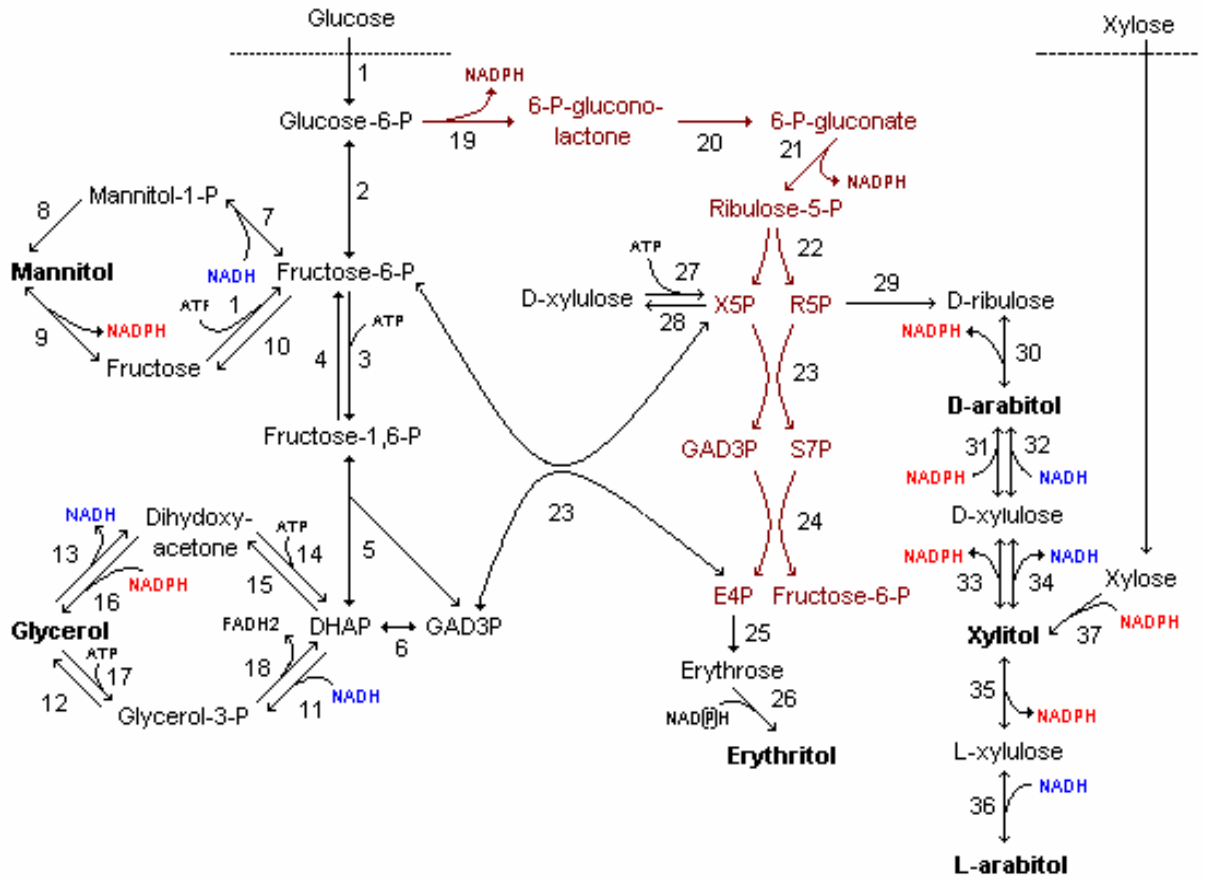



Figure 1 – Polyol anabolic and catabolic pathways in *A. niger*. 1. hexokinase, 2. glucose phosphate isomerase, 3. 6-phosphofructokinase, 4. hexose biphosphatase, 5. fructose biphosphate aldolase, 6. triose phosphate isomerase, 7. NAD-dependent mannitol-1-phosphate dehydrogenase (MPD), 8. mannitol-1-phosphate phosphatase, 9. NADP-dependent mannitol dehydrogenase, 10. fructose-6-phosphate phosphatase, 11. NAD-dependent glycerol-3-phosphate dehydrogenase (or DHAP reductase), 12. glycerol-3-phosphate phosphatase, 13. NAD-dependent glycerol dehydrogenase, 14. DHA kinase, 15. DHAP phosphatase, 16. NADP-dependent glycerol dehydrogenase (or NADPH-dependent DHA), 17. glycerol kinase, 18. FAD-dependent glycerol-3-phosphate dehydrogenase, 19. glucose-6-phosphate dehydrogenase, 20. lactonase, 21. 6-phosphogluconate dehydrogenase, 22. phosphoriboisomerase, 23. transketolase, 24. transaldolase, 25. erythrose-4-phosphate phosphatase, 26. erythrose reductase, 27. D-xylulose kinase, 28. D-xylulose-5-phosphate phosphatase, 29. D-ribulose-5-phophate phosphatase, 30. NADP-dependent D-ribulose reductase, 31. NADP-dependent D-xylulose reductase, 32. NAD-dependent D-xylulose reductase, 33. NADP-dependent xylitol dehydrogenase, 34. NAD-dependent xylitol dehydrogenase, 35. NADP-dependent L-xylulose reductase, 36. NAD-dependent L-arabitol dehydrogenase, 37. D xylose reductase.

In 1967, Lewis and Smith pointed out that polyols could (i) act as carbohydrate reserves, (ii) have a role in osmoregulation, (iii) store reducing power and (iv) function as translocatory compounds. During the last forty years, only few studies have dealt with the role of polyols in *Aspergillus spp.* and knowledge on their physiological role is still not clear.

Studies have confirmed that polyols can act as carbon storage compounds. Polyols are metabolised under starvation conditions (Corina and Munday, 1971; Dijkema et al.,

1985; Witteveen and Visser, 1995), and these storage compounds have been reported to appear in a consecutive manner, C6 polyols being synthesized first, thereafter C5 polyols and finally C4 polyols (Dijkema et al., 1985). Mannitol is found in high concentrations in conidia of some *Aspergilllus spp.* and is metabolised at a very early stage of the germination process (Horikoshi et al., 1965; Witteveen and Visser, 1995). Thus it was believed to be the main carbon storage compound in *Aspergillus* conidia. However, recent studies rejected this hypothesis and concluded that the main function of mannitol in conidia is to protect the spores against different stresses like high temperature, oxidative stress, freezing or lyophilisation (Ruijter et al., 2003).

The contribution of polyols to maintain the osmotic balance has been mainly studied in yeast where glycerol and to a lesser extent mannitol, sorbitol and xylitol can be accumulated to protect the cell against osmotic stress (Birkinshaw et al., 1931; Hohmann, 2002; Petrovska et al., 1999; Shen et al., 1999). Studies in *Saccharomyces cerevisiae* showed that the high osmolarity glycerol pathway (HOG) senses and signals osmotic changes, which leads to an increase in the polyol production, while the transmembrane flux is tightly controlled via an aquaglyceroporin Fps1p. Recent genome sequencing reveals homologues of Fps1p in other fungi but no functional transporter has been characterised (Hohmann, 2002). In *A. nidulans* and *A. niger*, glycerol and erythritol have been mentioned to play a role in osmoregulation (Beever and Laracy, 1986; Gutiérrez-Rojas et al., 1995MC; Witteveen and Visser, 1995) whereas in *Aspergillus oryzae* glycerol, erythritol and arabitol are involved in the response to osmotic stress (Ruijter et al., 2004). Moreover, these polyols help to maintain a higher intracellular osmolarity than in the environment and generate the tugor potential needed for growth (d'Enfert and Fontaine, 1997).

Polyols may play a role in balancing the cellular redox potential as the synthesis and catabolism of polyols generally involve the cofactors NADH and NADPH. But this role has not been proven in *Aspergillus spp.* in contradiction to yeast like *S. cerevisiae* where glycerol is produced as a response to high catabolic reduction of charge defined as the ratio [NADH]/([NADH]+[NAD]) (Costenoble et al., 2000). In the particular case of mannitol, theoretically, the enzymes present in *Aspergillus spp.* could allow the interconversion of fructose-6-phosphate and mannitol (Hult and Gatenbeck, 1978). This interconversion, known as the mannitol cycle would lead to generation of NADPH using NADH and ATP. Even though some studies indicate the presence of the mannitol cycle in *Alternaria alternata* (Hult and Gatenbeck, 1978), the mannitol cycle is generally not believed to be active. In 1969, Strandberg, showed the lack of coordinated change in the activities of two enzymes of the mannitol cycle, in response to a change in carbon source in *A. candidus*. In 1988, Sting et al. studied the influence of different carbon and nitrogen sources on the activity of the enzymes involved in the mannitol cycle, and they

concluded that in *A. nidulans*, the mannitol cycle is not playing a role in NADPH generation as there is no correlation between activities of the enzymes involved in the cycle and the need for NADPH. Recently, in 2004, Ruijter et al. showed that the use of nitrate as nitrogen source does not increase the expression of *mpdA* gene encoding for the NAD-dependent mannitol-1-phosphate dehydrogenase (MPD) in *A. niger N400*.

The possible role of polyols as translocatory compounds is speculative. If the transport were to be a proton-linked symport as in the case for pentitol in a number of fungi (Eddy, 1982) then, polyol excretion could be seen as a sink for protons and hereby could assist in regulating the cytoplasmic pH. Furthermore, polyols could act as "physiological buffer agents" by maintaining the appropriate redox state in the cytoplasm and by helping to maintain a suitable environment that ensures efficient enzyme activity (Jennings, 1984). However, if recent studies have characterised proton symporters for the uptake of polyols (Ferreira et al., 2005; Lages et al., 1999), polyol excretion is more likely to occur via facilitated diffusion through aquaglyceroporin (Hohmann, 2002). The MPD enzyme, involved in mannitol production, may play a role in the transfer of reducing equivalents between the cytoplasm and the mitochondrium (Singh et al., 1988).

Finally, another function of polyols and especially mannitol would be to prevent oxidative damage. This function has been suggested in the fungi *A. alternata* (Jennings et al., 1998), and *Cryptoccoccus neoformans* (Chaturvedi et al., 1996).

There is a discrepancy between the importance of polyols, one of the most abundant group of compounds in fungal mycelia (Blumenthal, 1976) and the knowledge we have on their function. The objective of this study is to gain further insight into the role of polyols in *A. niger* by studying their synthesis under different growth conditions, i.e. during growth on different carbon sources (glucose or xylose) and nitrogen sources (ammonium or nitrate) in batch cultures, where there is a dynamic shift from high to low concentrations of dissolved oxygen.

## 3. Materials and methods

### a. Microorganism

In this study, the *BO1* strain of *A. niger* was used (Pedersen et al., 2000). The strain was kindly donated by Novozymes, and is a glucoamylase over-producer. The strain contains one copy of the glucoamylase gene. The organism was maintained as frozen spore suspensions, at -80°C, in 20% glycerol.

### b. Media

The composition of the batch cultivation medium was: 100 g/L glucose.$H_2O$, 12 g/L $NH_4Cl$, 3.0 g/L $KH_2PO_4$, 1.0 g/l $MgSO_4.7H_2O$, 1.0 g/l NaCl, 0.25 g/L $CaCl_2$.2H2O, 0.5 mL/L antifoam (antifoam 204, Sigma), 14.3 mg/L $ZnSO_4.7H_2O$, 2.5 mg/L $CuSO_4.5H_2O$, 0.50 mg/L $NiCl_2.6H_2O$, 7 mg/L $MnCl_2.H_2O$ and 13.8 mg/L $FeSO_4.7H_2O$. When xylose was used as the carbon source, glucose.$H_2O$ was replaced with 90 g/L xylose. When nitrate was used as the nitrogen source, $NH_4Cl$ was replaced with 19 g/L $NaNO_3$.

### c. Cultivation conditions

A defrosted spore solution was inoculated on LCS plates containing: 15.75 g/L Lactose.$H_2O$, 5 g/L Bacto-Soytone, 4 g/L NaCl, 0,5 g/L $MgSO_4.7\ H_2O$, 0.6 g/L $KH_2PO_4$, 2.5 mL/L Corn Steep Liquor, 0.005 g/L $FeCl_3$, 0.002 g/L $CuSO_4$, 20 g/L Agar adjusted to a pH of 5.5. The spores were propagated at 30°C for 7 days and they were harvested by adding a suitable amount of 0.01% Tween 80. They were successively centrifuged (4000 rpm, 10 minutes) and washed three times in the same volume of NaCl 0.9%. The fermentors were inoculated with this suspension to obtain a final concentration of $3.10^8$ spores/L. The cultivations were run in 5 litres reactors equipped with pH and temperature control. The Dissolved Oxygen Tension (DOT) was measured with an oxygen probe (Mettler Toledo sterilizable/autoclavable sensors). Inlet air was controlled with a mass flowmeter. The composition of the inlet and outlet gas was analysed by a gas analyser (1311 Fast response Triple gas, Innova combined with Multiplexer controller for Gas Analysis MUX100, B. Braun Biotech International).

The initial set up of the fermentors was: a pH of 3, an aeration rate of 0.04 vvm (volume per volume per minute) and a stirring speed of 100 rpm, in order to avoid the formation of pellets and loss of the hydrophobic spores from the medium to the head-space surface of the bioreactor. After germination, the pH was gradually stepped up to 4.5, the aeration rate to 0.2 vvm and the stirring speed to 900 rpm at which values all cultivations were carried out. The low aeration rate used in this study was in order to obtain DOT limitation. The temperature was kept at 30°C during all the fermentations.

### d. Sampling

For biomass measurements, the samples were filtered (paper filters, Whatmann, cat n°1001070), washed with 0.9 % NaCl, dried at 105°C for 24-48 h and cooled down for at least 2 hours in a desiccator. The filtrate was filtered once more (acetate, pore size 0.22 µm, Cameo 25GAS) and frozen for later HPLC analysis.

### e. Quantification of sugars and extracellular metabolites

Culture samples were analysed for quantification of sugar (glucose or xylose) and identification of extracellular metabolites (acetate, citrate, ethanol, fumarate, malate, pyruvate and succinate). This was done with an isocratic HPLC method, using an aminex HPX-87H column (Biorad). The column was eluted at 60°C with 5 mM $H_2SO_4$ at a flow rate of 0.6 mL/min. Metabolites were detected with both a refractive index detector and a UV detector.

### f. Quantification of polyols

Polyols (arabitol, erythritol, glycerol, mannitol and xylitol) were quantified using an isocratic HPLC method (Dionex DX500). The column (CarboPac MA1) was eluted at 60°C with 612 mM NaOH at a flow rate of 0.4 mL/min and an electrochemical detector (ED 40) was employed. This method also enables quantitative analysis of trehalose. However the trehalose concentration did not exceed 0.25 g/L and this compound has therefore not been included in the further analysis.

### g. Yield determination

The yields of biomass, metabolites and $CO_2$ have been determined by plotting the concentrations of biomass, metabolites and $CO_2$ against the concentration of glucose. For each fermentation, two linear correlations were obtained: one for the exponential phase and one for the oxygen limitation phase. The different yields were calculated using a linear regression applied to the both phases. They are presenting in table 1.

## 4. Results

### a. Influence of the availability of oxygen

All the fermentations were characterized by a lag phase, a growth phase and an oxygen limitation phase. Figure 2 represents a typical fermentation profile: for this fermentation the carbon source was xylose and the nitrogen source was nitrate. The production of biomass during the exponential phase resulted in an increased viscosity of the medium and hence a decrease in the mass transfer, especially oxygen transfer (Barberel and Walker, 2000). The consequence was a decrease in the Dissolved Oxygen Tension (DOT) from 100% to 1%. When the DOT was below the critical value of 20%, the oxygen uptake rate decreased (Figure 2a). The decrease of the oxygen uptake rate, followed by a decrease in the $CO_2$ production rate, characterized the beginning of the oxygen limitation phase. The glucose consumption rate was even more affected by the oxygen limita-

tion (data not shown) and the consequence was an increase in the yield of $CO_2$ in Cmol per Cmol carbon (Table 1).

| Fermentation Phase | | Glucose-Ammonium growth | Glucose-Ammonium $O_2$ limitation | Glucose-Nitrate growth | Glucose-Nitrate $O_2$ limitation | Xylose-Ammonium growth | Xylose-Ammonium $O_2$ limitation | Xylose-Nitrate growth | Xylose-Nitrate $O_2$ limitation |
|---|---|---|---|---|---|---|---|---|---|
| Biomass | | 0.43 | 0.27 | 0.40 | 0.25 | 0.48 | 0.26 | 0.33 | 0.20 |
| Polyols | Glycerol | 0.09 | 0.08 | 0.08 | 0.09 | 0.02 | 0.06 | 0.01 | 0.06 |
| | Erythriol | 0.00 | 0.02 | 0.01 | 0.03 | 0.02 | 0.04 | 0.03 | 0.05 |
| | Mannitol | 0.00 | 0.09 | 0.00 | 0.10 | 0.00 | 0.04 | 0.00 | 0.04 |
| | Arabitol | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 |
| | Xylitol | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.05 | 0.03 | 0.06 |
| Organic acids | Succinate | 0.00 | 0.02 | 0.01 | 0.03 | 0.02 | 0.06 | 0.04 | 0.07 |
| | Citrate | 0.00 | 0.00 | 0.02 | 0.01 | 0.01 | 0.01 | 0.00 | 0.01 |
| | Pyruvate | 0.00 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| $CO_2$ | | 0.33 | 0.44 | 0.47 | 0.49 | 0.41 | 0.49 | 0.49 | 0.53 |
| C Balance | | 0.85 | 0.94 | 1.00 | 1.00 | 0.96 | 1.00 | 0.94 | 1.02 |

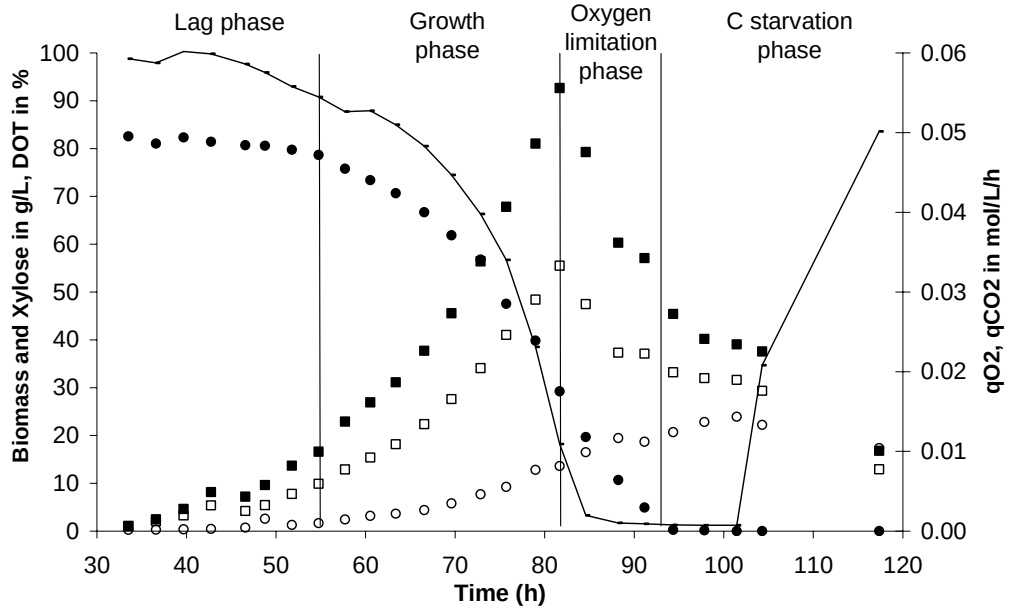
Table 1 – Carbon balance in Cmol per Cmol carbon source.

Other consequences of the shift to oxygen limitation were a decrease in the yield of biomass in Cmol per Cmol carbon (decrease of 40% in average) and an increase in the production yield of several polyols (increase of 60% in average). The polyols produced were glycerol, mannitol, erythritol and arabitol. Although the production of arabitol was observed for each fermentation, its production yield was as low as 0.005 Cmol/Cmol carbon source for most of the conditions, and it is therefore not considered in the further discussion. Xylitol was only produced if the carbon source was xylose. During the growth phase, glycerol was the main polyol produced and represented up to 9% of the carbon consumed, except for the fermentation using xylose as carbon source and nitrate as nitrogen source. In this case, the main polyols produced were xylitol and erythritol and their production represented 6% of the carbon consumed. Mannitol was only produced in oxygen limitation and represented up to 10% of the carbon consumed. It was the main polyol produced during oxygen limitation in the fermentations with glucose as carbon source. When xylose was used as carbon source the main polyols produced during oxygen limitation were glycerol and erythritol representing respectively 6% and 5% of the carbon consumed (Table 1).

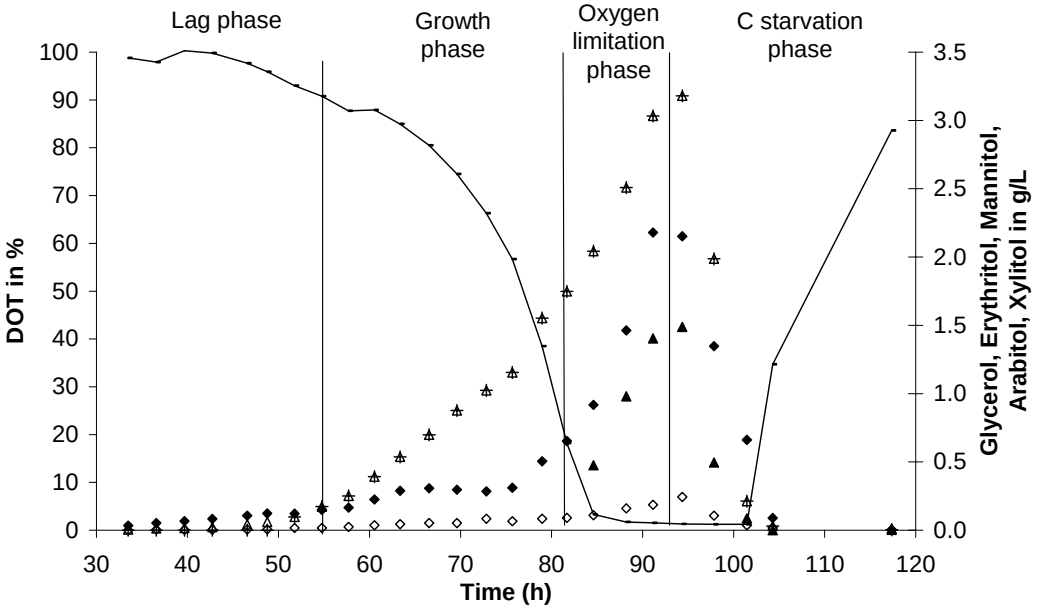
Independently of the carbon source and the nitrogen source used, production yields of mannitol, erythritol, arabitol and xylitol increased when there was a shift to oxygen limitation (Table 1). The main organic acid produced was succinate and the yield was increased during oxygen limitation. Citrate and pyruvate were also produced but in low quantities and the maximum yields (0.02 Cmol/Cmol carbon) were reached during the exponential phase on growth on glucose as carbon source and nitrate as nitrogen source. Interestingly, ethanol and acetate were not produced by this strain even during severe oxygen limitation. The carbon balance of these fermentations closed with a maximum error of 6% except for the growth phase of the fermentation using glucose and ammonium for which the carbon balance closed at 85%. This suggests that the

main products of these fermentations have been quantified. The glucoamylase production by *A. niger BO1* in these cultivations conditions should not affect the carbon balance. Indeed, Pedersen et al. (2000) have shown that during similar batch cultivation conditions, using 20 g/L of glucose, the glucoamylase yield was 0.015 g/g glucose. In the present study, considering the high glucose concentration, which inhibits the production of glucoamylase, the glucoamylase yield is expected to be lower than 0.015 g/g glucose, and the amount of carbon directed towards glucoamylase production can therefore be neglected. After depletion of the initial carbon source, glucose or xylose, the polyols and the organic acids were consumed (Figure 2b and c). After consumption of all the carbon sources the DOT increased due to cell death.

**a**




**b**



**c**

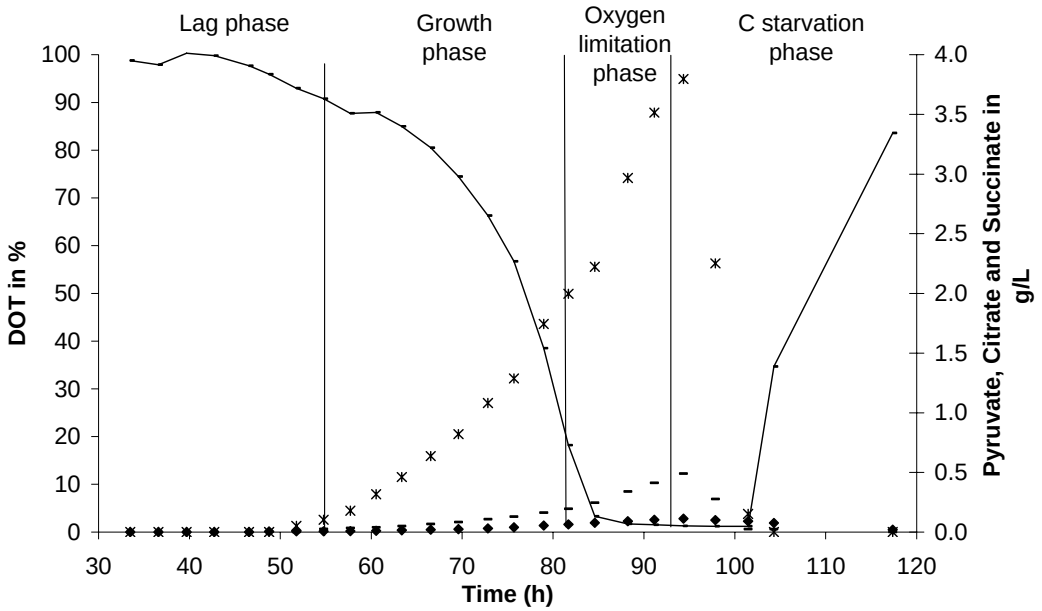
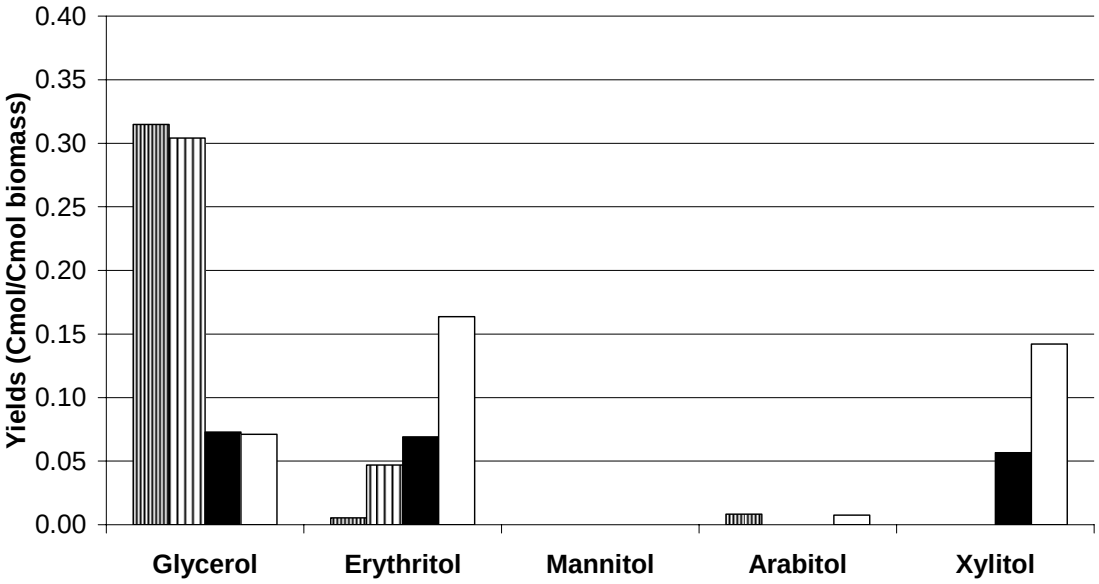



Figure 2 – Profile of the fermentation using xylose as carbon source and nitrate as nitrogen source. (a) Biomass (○), xylose (●), DOT (−), $qO_2$ (□) and $qCO_2$ (■). (b) DOT (-), mannitol (▲), erythritol (Δ), glycerol (♦) arabitol (□) and xylitol (+). (c) DOT (−), pyruvate (♦), citrate (—) and succinate (ж).

### b. Influence of the nitrogen source.

Two nitrogen sources were investigated: ammonium and nitrate. The influence of the nitrogen source on the polyol pattern is shown in figure 3. Independently of the carbon source and the oxygen availability, the use of nitrate instead of ammonium increased the specific yields of erythritol and xylitol. At oxygen-limiting conditions, independently of the carbon source used, the consequences of using nitrate were an increase in the specific yields of glycerol and mannitol and a decrease in the specific yield of arabitol. The decrease in the specific yield of arabitol might not be significant, since the concentrations obtained were low. Concerning the production of organic acids, the yield of succinate increased in presence of nitrate. Moreover, the use of nitrate led to a decrease in the biomass yield and to an increase in the $CO_2$ yield (Table 1).

**a**




**b**

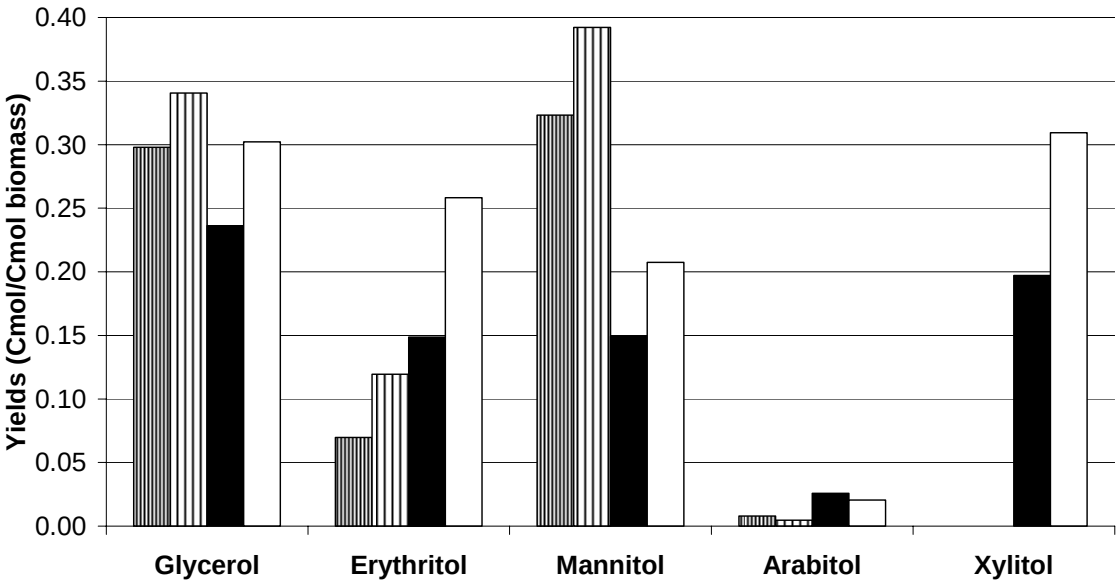



Figure 3 – Yields in Cmol per Cmol of biomass for the fermentations using glucose and ammonium ( ▨ ), glucose and nitrate ( ▥ ), xylose and ammonium ( ■ ), xylose and nitrate ( □ ). (a) yields during the growth phase. (b) yields during the oxygen limitation phase.

### c. Influence of the carbon source

The high carbon source concentration used in this study increased the osmotic pressure of the medium and led to a long lag phase, lasting more than 38 hours. The use of xylose instead of glucose resulted in a change in the polyol pattern (Figure 3). Independently of the nitrogen source and the oxygen availability the specific yields of glycerol and mannitol decreased and the specific yields of erythritol, arabitol and xylitol increased during growth on xylose compared with during growth on glucose. Further-

more, during growth on xylose, the yield of succinate and $CO_2$ increased while the biomass yield decreased. There is one exception. During the growth phase, the biomass yield of the fermentation run with glucose and ammonium is lower than the one obtained for the fermentation using xylose and ammonium. This observation may indicate that, during the growth phase, a part of the carbons missing in the carbon balance of the fermentation run with glucose and ammonium corresponds to carbons oriented to the production of biomass.

## 5. Discussion

### a. Influence of the oxygen availability

In *A. niger,* oxygen plays an important role in the energy metabolism as it serves as electron acceptor in the oxidative phosphorylation. Here NADH is oxidized to NAD and the electrons are, via the electron transport chain, transferred to oxygen, which serves as the terminal electron acceptor. Thus, a decrease in the oxygen uptake leads to an accumulation of NADH and also to a decrease in the ATP synthesis. Therefore, a limitation in the oxygen uptake may cause a global effect on the metabolism as it may result in an effect on all enzymes that use or produce NADH and ATP. Particularly, it may affect the activity of enzymes involved in the tricarboxilic acid (TCA) cycle, which could be the cause of the increase in succinate production during oxygen limitation. Moreover, the accumulation of NADH may activate NADH-dependent enzymes in order to facilitate the reoxidation of this cofactor. This could explain the accumulation of mannitol during oxygen limitation, as its production involves MPD which regenerates NAD. Mannitol can also be produced via the fructose-6-phosphate phosphatase and the NADP-dependent mannitol dehydrogenase, but the study of Ruijter et al. (2003) has shown that the MPD and the mannitol-1-phosphate phosphatase form the main pathway for the mannitol biosynthesis in *A. niger*. Furthermore, in 1931, Birkenshaw et al. studied the mannitol production in different *Aspergillus spp.* and they showed that a decrease in the aeration rate increases the production yield of mannitol.

Oxygen plays a function as electron acceptor in biosynthesis of essential biomass components. Thus, yeast and fungi require oxygen for biosynthesis of unsaturated fatty acids and sterols. At low oxygen concentration, the biosynthesis of these essential components may be affected (Barberel and Walker, 2000). This phenomenon, associated to the increasing ratio of NADH/NAD and the decrease in the ATP synthesis, could explain the decrease in the specific growth rate during oxygen limitation.

The increase in the $CO_2$ production yield was a consequence of carbon flux deviation to the PP pathway and/or to the citrate cycle, which are the two main pathways producing $CO_2$. A deviation of carbon flux towards the PP pathway could explain the increase of

the production yield of erythritol and arabitol during limited oxygenation. A study on citric acid production in *A. niger* (Röhr et al., 1987) pointed out that erythritol was accumulated in the late stage of fermentation, when the demand for the biosynthetic intermediates was low and the PP pathway was still active accounting for 10-20% of the sugar metabolised. In this case, the percentage of sugar converted to erythritol was 10%. Based on these findings, it was concluded that the accumulation of erythritol was due to excess activity of the PP pathway in excess compared with the cellular demand for the pathway intermediates (Röhr et al., 1987). Recently, Poulsen et al. (2005) have also observed high PP pathway enzyme activities during stationary phase in *A. niger*. In the present work, the production yields of erythritol and arabitol increased during oxygen limitation when the biomass production was low and the production of $CO_2$ was high. Consequently, there is a high probability that the production of these polyols was a result of a deviation of the carbon flux to the PP pathway combined with a decrease of the need for biosynthetic intermediates.

In contradiction to the above, Dijkema et al. (1985) have shown that in *A. nidulans* limited oxygenation could lead to low levels of arabitol, erythritol and glycerol and that an increase in the oxygen uptake could lead to an overflow of reduced metabolites from both the PP pathway (erythritol and arabitol) and the glycolysis (glycerol). These differences could be due to the use of different strains or to the use of completely different cultivation conditions (shake flask cultures with different medium and growth parameters in Dijkema et al. study). Particularly the osmotic pressure was different in the two studies. Indeed, in the present work, all the fermentations have been performed with high sugar concentrations, which increase the osmotic pressure of the medium. Glycerol production and to a lesser extent, erythritol and xylitol production, started in the exponential phase and may have been produced due to this high osmotic pressure. Glycerol and erythritol have been found to be produced as a defence mechanism against osmotic stress in *Aspergillus sp.* (Beever and Laracy, 1986; Ruijter et al., 2004; Witteveen and Visser, 1995). These osmolytes are mainly accumulated intracellularly, but a part of them is released into the medium (Blomberg and Adler, 1989). Glycerol may also play another role. Indeed, two pathways can lead to the production of glycerol: one via the NAD-dependent glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate phosphatase and another one via DHAP phosphatase and the NADP-dependent glycerol dehydrogenase. The flux through either route depends on the phoshatase activities but mainly on the activity of the NAD-dependent glycerol-3-phosphate dehydrogenase and on the activity of the NADP-dependent glycerol dehydrogenase. Schuurink et al. (1990) showed that the activity of NADP-dependent glycerol dehydrogenase was strongly dependent of the anabolic reduction charge. Hondmann (1994) concluded that a high anabolic reduction of charge, meaning a high flux through the PP pathway favours the

route via DHA, while a high flux through the glycolysis and thus a high catabolic charge, favours the route via glycerol-3-phosphate. During oxygen limitation, there is an accumulation of NADH in the cell, which would favour the glycerol production via glycerol-3-phosphate. Moreover, if the hypothesis made before, concerning a deviation of the carbon flux through the PP pathway at oxygen limitation were true, this would imply a high anabolic reduction charge and thus an activation of the NADP-dependent glycerol dehydrogenase. Thereby, both biosynthetic pathways could be active at oxygen-limiting conditions. Then the glycerol production could be explained by a high osmotic pressure in the first part of the fermentation and by a high catabolic and maybe anabolic reduction of charge during oxygen limitation.

Concerning xylitol, this polyol is mainly produced when xylose is used as carbon source. Xylose is metabolised via xylitol, xylulose and xylose-5-phosphate. The step xylitol to xylulose is mainly catalysed by the NAD-dependent xylitol dehydrogenase, which is sensitive to the catabolic reduction charge and inhibited by an accumulation of NADH (Winkelhausen and Kuzmanova, 1998; Witteveen et al., 1994). Consequently, the increasing accumulation of xylitol, during oxygen limitation, was most likely due to a decrease of the NAD-dependent xylitol dehydrogenase activity.

### b. Influence of the nitrogen source

The use of nitrate as nitrogen source requires reduction of nitrate to ammonium. This biosynthetic pathway requires the use of two moles of NADPH per mole of nitrate consumed, and it represents a major drain for this cofactor. NADPH is mainly produced in the PP pathway. For each glucose-6-phosphate entering the PP pathway two NADP molecules are reduced by the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. When nitrate is used as nitrogen source, an increase of these two enzyme activities has been noticed in *A. nidulans* and *A. niger* (Habison et al., 1983; Hankinson and Cove, 1972; Kinghorn and Unkles, 1994; Poulsen et al., 2005; Singh et al., 1988). More specifically, Poulsen et al. (2005) have shown that, in *A. niger*, the activities of three enzymes belonging to the PP pathway increase when nitrate is added to the medium: glucose-6-phosphate dehydrogenase, 6 phosphogluconate dehydrogenase and transketolase. This phenomenon would most likely result in an increased flux through the PP pathway (Pedersen et al., 1999) and could explain the increase of the erythritol and xylitol production when nitrate is used as nitrogen source, as these two compounds are synthesized from intermediates of the PP pathway. Thus, the increased production of erythritol and xylitol could be a consequence of a metabolic overflow in the PP pathway due to the increased requirement for NADPH during growth on nitrate. The increasing $CO_2$ yield when nitrate is used as nitrogen source may also be linked to the increasing flux through the PP pathway.

Other enzymes can be involved in the production of NADPH: isocitrate dehydrogenase, malate dehydrogenase (both presence and absence of this enzyme has been reported in *A. niger* (Hankinson and Cove, 1972)) and also NADP-dependent mannitol dehydrogenase. As mentioned in the introduction, different opinions have been raised concerning the implication of the NADP-dependent mannitol dehydrogenase in the regeneration of NADPH. However, a study in *Aspergillus parasiticus* (Niehaus and Jiang, 1989) has shown that the use of nitrate as nitrogen source increases the activity of the NADP-dependent mannitol dehydrogenase and the activity of the MPD. The consequence would be an increase in the activity of the mannitol cycle in presence of nitrate. Moreover, they specify that the activity of MPD, involved in the production of mannitol, increased 10 times and the activity of the NADP-dependent mannitol dehydrogenase, involved in the degradation of mannitol, increased 3 times. Unfortunately, Niehaus and Jiang (1989) did not measure the mannitol concentration, but this increase of enzymatic activities could lead to an augmentation of mannitol accumulation and could explain the increase in mannitol production observed here during growth on nitrate.

Another possible explanation for the increase of the mannitol production is the effect of $NH4^+$ on the enzyme phosphofructokinase. This cation, irrespectively of the anion associated, is a powerful activator of phosphofructokinase (Habison et al., 1983). More precisely, it increases the affinity of the phosphofructokinase for its substrate: fructose-6-phosphate. Thereby it decreases the availability of fructose-6-phosphate for the MPD leading to the production of mannitol. Thus, the use of $NH4^+$ may have contributed to the lower mannitol production during growth on ammonia.

Moreover, during growth on nitrate, the increase in the polyol specific yield in Cmol/Cmol biomass is also linked to the decrease in the biomass yield. The decrease in the biomass is due to a loss of carbon as $CO_2$ and to unfavourable energetic conditions as the demand for NADPH is high. The changes in the anabolic reduction charge associated with a decrease in the need of components for biomass synthesis led to an accumulation of metabolites from the TCA cycle.

### c. Influence of the carbon source

Xylose is metabolised via xylitol, xylulose and xylulose-5-phosphate. In 1994, Witteveen et al. have shown that the conversion of xylitol to xylulose is mainly done by the NAD-dependent xylitol dehydrogenase. They have characterized this enzyme and they predicted an accumulation of xylitol *in vivo* due to the low Vmax of this enzyme. This could explain the accumulation of xylitol when xylose is used as carbon source.

With xylose as the carbon source, the entry point of the metabolism is the PP pathway, downstream of the oxidative part including two NADPH forming reactions. To produce NADPH via the PP pathway under these conditions, there needs to be an important

backflux from fructose-6-phosphate to glucose-6-phosphate and back into the PP pathway. Moreover, the conversion of xylose to xylitol involves a molecule of NADPH and increased the demand for this cofactor. At these conditions, the carbon flux was mainly oriented towards the PP pathway and the production yields of the polyols produced in the PP pathway increased (erythritol, arabitol, xylitol) while the production yields of the polyols produced in the Embden-Meyerhof-Parnas (EMP) pathway decreased (mannitol, glycerol). This re-direction of the flux through the PP pathway increased the $CO_2$ yield when xylose was used as carbon source. Moreover, the activity of the TCA cycle seems to have been affected as the accumulation of succinate increased.

During the growth phase, on xylose, the low glycerol yield was associated with a high yield of erythritol and xylitol. These results suggest that glycerol, erythritol and xylitol, contribute to maintain the osmotic balance. The ratio between the three polyols is depending on the carbon flux in the EMP pathway and in the PP pathway. When the flux via the EMP is high, the production of glycerol is favoured over the production of erythritol and xylitol and vice versa.

## 6. Conclusion

Under the conditions studied, polyols were the most abundant compounds produced. They represented up to 22% of the carbon consumed. The quantity produced and the polyol pattern depended strongly on the environmental conditions. This is due to a complex regulation of polyol production and to the fact that each polyol can fulfil different functions. This study indicates different function of the polyols.

First, polyols have a role as carbohydrate reserves. Erythritol, xylitol and arabitol are produced as carbon storage compounds when the flux through the PP pathway exceeds the need in ribulose-5-phosphate for the biomass synthesis. At the later stage of the fermentation, all polyols are consumed as soon as the main carbon source, glucose or xylose, is depleted.

Secondly, this work suggests that, in *A. niger*, glycerol, erythritol and xylitol are involved in osmoregulation. The proportion between these polyols is depending on the carbon flux in the EMP pathway and PP pathway.

A third role pointed out by this work is the role in balancing the cellular redox potential. During oxygen limitation, NADH is partly reoxidized via the MPD and the consequence is an accumulation of mannitol. More precisely, mannitol seems to be the main compound involved in NADH reoxidation during oxygen limitation. This study shows a main difference between filamentous fungi like *A. niger* and yeast like *S. cerevisiae* as in yeast an accumulation of NADH in the cell leads mainly to the production of glycerol and ethanol and not mannitol. The role of mannitol in the generation of NADPH has been

suggested but not proved. At these fermentation conditions, another metabolite is produced: succinate. The production of succinate is enhanced at oxygen limitation, particularly during growth on nitrate and during growth on xylose, when the flux in the TCA cycle is low due to an accumulation of NADH in the cell and to a reorientation of the carbon flux trough the PP pathway from glyceraldehyde-3-phosphate to glucose-6-phosphate.

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# III. Physiology of *Aspergillus niger* in oxygen-limited continuous cultures: influence of aeration, carbon source concentration and dilution rate.

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## 1. Abstract

Oxygen availability is important for industrial fermentations with *Aspergillus niger*. We therefore studied the effect of varying oxygen availability, at different carbon source concentrations and at different specific growth rates, on the metabolism of *A. niger,* using continuous cultures. The results show that there is an increase in the production of TCA cycle intermediates at low oxygen availability. Indeed, under these conditions, a decrease of the mitochondrial respiratory chain activity leads to an accumulation of NADH and to a decreased ATP production, which can influence the intracellular pH, leading to production and excretion of organic acids. Moreover, this work demonstrates that the production of mannitol, involving the reoxidation of NADH, is the main cellular response to balance the ratio NADH/NAD at low oxygen availability. Mannitol production is also coupled to low specific growth rate, which suggests a control of carbon catabolite repression on the mannitol pathway. The roles of two other polyols, erythritol and glycerol, have also been investigated. Both compounds are accumulated intracellularly, at high osmotic pressure, in order to restore the osmotic balance on both sides of the cell wall. However, we showed that the efficiency of this system is affected by a leakage of polyols through the membrane.

## 2. Introduction

During aerobic microbial growth, oxygen plays an important role as the terminal electron acceptor in the respiratory chain. It allows the reoxidation of NADH and $FADH_2$ and when electrons traverse the electron transport chain the Gibbs free energy is used to transport protons against an electrochemical gradient, which subsequently drives the production of ATP via the $F_0F_1$-ATPase. In submerged fermentations, the respiration rate is directly affected by the oxygen transfer into the medium. Several resistances are encountered as oxygen is transferred from the gas phase to the respiratory chain. The main resistance is located at the interface between gas and liquid phases (Kobayashi et al., 1973), and the degree of resistance depends on the physical properties of the medium (Barberel and Walker, 2000). In the particular case of fermentations with filamentous fungi, the interactions between the freely dispersed hyphae result in a network formation, leading to an increase in medium viscosity. The impact of these interactions is increasing as the biomass concentration increases (Bocking et al., 1999; Roels et al., 1974). The main consequences are an increase in the transfer resistance and a decrease in the oxygen transfer resulting potentially to periods of oxygen limitation (Diano et al., 2006). So far, data on the consequences of oxygen limitation on the metabolism of filamentous fungi in such cultivations are scarce. A previous study using *Aspergillus niger*

*BO1* has shown that, in batch cultivations, the main consequence of oxygen limitation is the production of mannitol (Diano et al., 2006). The MPD, involved in the production of mannitol-1-phosphate from fructose-6-phosphate, seems to be the main enzyme involved in the reoxidation of NADH when the final electron transport acceptor, oxygen, is limiting. Moreover this work has shown that oxygen limitation leads to an increase in the production of organic acids such as citric and succinic acids (Diano et al., 2006). In this study, we used continuous cultures, as they enable a far better control of the environmental conditions than batch and fed batch cultures, and provide a good setting to study the influence of a single parameter on the cell metabolism. The metabolism of *A. niger* was first studied under different conditions of aeration. We then investigated the effect of the specific growth rate and glucose concentration under oxygen-limiting conditions. Furthermore, the physiology of *A. niger* was studied under extreme oxygen-limiting conditions, using oxygen-limited continuous cultivations combined with a step down in oxygen supply.

## 3. Materials and methods

### a. Microrganism

In this study, the *BO1* strain of *A. niger* was used (Pedersen et al., 2000). The strain was kindly donated by Novozymes. The organism was maintained as frozen spore suspensions, at -80°C, in 20% glycerol.

### b. Media

#### *Preparation of inoculum*

A defrozen spore solution was inoculated on LCS plates containing: 15.75 g/L Lactose·$H_2O$, 5 g/L Bacto-Soytone, 4 g/L NaCl, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L $KH_2PO_4$, 2.5 mL/L Corn Steep Liquor, 5.0 mg/L $FeCL_3$, 2.0 mg/L $CuSO_4$, 20.0 g/L Agar Agar and adjusting to a pH of 5.5.

#### *Batch cultivations*

The composition for the batch cultivation medium was: 8 g/L glucose monohydrate, 1.5 g/L $KH_2PO_4$, 7.3 g/L $(NH_4)_2SO_4$, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L NaCl, 0.1 g/L $CaCL_2 \cdot 2H_2O$, 7.2 mg/L $ZnSO_4 \cdot 7H_2O$, 1.3 mg/L $CuSO_4 \cdot 5H_2O$, 0.3 mg/L $NiCL_2 \cdot 6H_2O$, 3.5 mg/L $MnCL_2 \cdot H_2O$, 6.9 mg/L $FeSO_4 \cdot 7H_2O$, 40 mg/L yeast-extract and 0.1 mL antifoam (antifoam 204, organic, Sigma). The yeast-extract was added, after autoclaving the medium, using a sterile filter (acetate, pore size 0.22 µm, Cameo 25GAS). To study the influence of glucose concentration, media containing 20 g/L or 50 g/L of glucose mono-

hydrate were used. To study the influence of osmotic pressure the concentration of NaCl was increased to 0.5 M and 1 M.

***Continuous cultures***

The feed medium for the continuous cultivation contained the following chemicals: 8 g/L glucose monohydrate, 0.75 g/L $KH_2PO_4$, 2.5 g/L $(NH_4)_2SO_4$, 1.0 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L NaCl, 0.1 g/L $CaCL_2 \cdot 2H_2O$, 7.2 mg/L $ZnSO_4 \cdot 7H_2O$, 1.3 mg/L $CuSO_4 \cdot 5H_2O$, 0.3 mg/L $NiCL_2 \cdot 6H_2O$, 3.5 mg/L $MnCL_2 \cdot H_2O$, 6.9 mg/L $FeSO_4 \cdot 7H_2O$ and 1.0 mL antifoam (antifoam 204, organic, Sigma). To study the influence of glucose concentration, media containing 20 g/L or 50 g/L glucose monohydrate were used. To study the influence of osmotic pressure the concentration of NaCl was increased to 0.5 M and 1 M.

### c. Cultivations

***Preparation of inoculum***

The spores were propagated at 30°C for 7 days and were harvested by adding a suitable amount of 0.01% Tween 80. They were successively centrifuged (4000 rpm, 10 minutes) and washed 3 times in the same volume of NaCl 0.9%.

***Batch cultivations***

2 L Braun bioreactors were inoculated with a suitable amount of spore solution in order to obtain a final concentration of $3.10^8$ spores/L. The bioreactors were equipped with pH and temperature control. The temperature was kept at 30°C.

The initial set up of the bioreactors was: a pH of 3, an aeration rate of 0.04 vvm (volume per volume medium per minute) and a stirring speed of 100 rpm in order to avoid the formation of pellets and loss of the hydrophobic spores from the medium to the head-space surface of the bioreactor. After germination, the pH was gradually stepped up to 4.5, the aeration rate to 0.875 vvm and the stirring speed to 700 rpm at which values all cultivations were carried out. Under these cultivation conditions, the fungus grew as freely dispersed hyphae. No pellet was observed during all cultivations. To study the influence of aeration, the level of atmospheric air sparged into the bioreactor was de-creased from 100% to 50%, 25% and 12.5% by mixing the inlet air (78% nitrogen, 21% oxygen) with nitrogen gas (100% nitrogen, UN 1066, AGA GAS AB). The rates of air and nitrogen sparged into the fermentor were controlled precisely using mass flow controllers (EL-FLOW mass flow meters/controllers, Bronkhost Hi-Tec). The outlet gas was cooled to 5°C and the $CO_2$ content was analyzed using a gas analyzer (Inova, Denmark).

***Continuous cultures***

The feed was started in the late exponential phase. All continuous cultures were mass controlled and the dilution rate was controlled to 0.10 $h^{-1}$ or 0.05 $h^{-1}$. The cultivation was assumed to be in a physiological steady-state when no significant variation in dry weight, gas composition and extracellular metabolites was observed during 3 retention times. To avoid wall growth the part between the medium and the top of the bioreactor was cooled to 5°C and the glass part of the reactor was treated with the anti-adhesive agent sigmacote (Sigma-Aldrich).

***Oxygen step down experiments***

Oxygen-limited continuous cultures were carried out at a dilution rate of 0.05 $h^{-1}$ using 8 g/L of glucose monohydrate as carbon source. The air percentage of the inlet gas was first set up to 12.5% by adding a suitable amount of nitrogen. Once the steady state was reached, the air percentage was dropped either to 6.250% or to 3.125% depending on the experiments.

### d. Analytical methods

***Biomass dry weight***

For biomass measurements, defined volumes of broth (around 10 mL) were filtered on pre-weighted filters (Pall corporation, 47 mm, pore size 0.45 µm). The filters were then successively washed with 0.9% NaCl, dried in a microwave for 10 minutes at 135 W, cooled down for at least 2 hours in a desiccator and weighted.

***Quantification of glucose and extracellular metabolites***

The amounts of extracellular glucose, organic acids (acetic, citratic, fumaric, malic, pyruvic and succinic acids) and ethanol were analyzed using a high-performance liquid chromatography (HPLC) system. An isocratic method was used. An ion-exclusion column (Aminex HPX-87H) was eluated at 60°C with 5 mM $H_2SO_4$ at a flow rate of 0.6 mL/min followed by two detectors: a Waters 410 differential refractometer and a Waters 486 absorbance detector spectrophotometer at 210 nm. No ethanol or acetic acid were detected under these conditions of cultivation.

Polyols (arabitol, erythritol, glycerol, mannitol and xylitol) were quantified using an isocratic HPLC method (Dionex DX500). The column (CarboPac MA1) was eluted at 60°C with 612 mM NaOH at a flow rate of 0.4 mL/min and an electrochemical detector (ED 40) was used. No xylitol was detected under these conditions of cultivations. This method also enables quantitative analysis of trehalose. However, very low amounts of threalose were detected.

Moreover, during these continuous cultures, between 91% and 100% of the carbon consumed were recovered, indicating that the main carbon compounds produced were taken into account.

### e. Sampling of intracellular polyols

The method used was a modification of the method described by Witteveen et al. (1994). The biomass samples, containing about 200 mg dry weight, were filtered using miracloth (Calbiochem), frozen in liquid nitrogen and stored at -80°C until use.

### f. Extraction of intracellular polyols

The method to extract intracellular polyol from the mycelium was a modification of the method described by Witteveen et al. (1994). To the frozen sample 10% (v/v) ice-cold perchloric acid was added, approximately 3 mL per 100 mg mycelium. The sample was then shaken at 300 rpm and at 0-4°C, using a shaking table (Lka Labortechnik), during 1.5 h, until homogenisation. The sample was then filtered by vacuum filtration and washed with mQ water (one volume per volume of perchloric acid). Filters were used for dry weight determination and the filtrates were neutralised using a saturated solution of $KHCO_3$ before being centrifuged at 10.000 rpm for 15 minutes at 0°C. The supernatants containing the intracellular polyols were stored at -80°C until further analyses on HPLC (as described above for the extracellular polyols).

### g. Morphological analyses

The analysis of fungal morphology was realised as described by Haack et al. (2006). For each sample a minimum of 10 pictures were taken and on each picture the diameter of hyphae was measured 3 to 4 times. In total, an average of 40 values were collected per sample, on which average and standard deviation were determined. The average and standard deviation of the hyphal growth per tips were also determined using 40 measurements. Comparison between 2 given conditions was carried out using a Student's t-test with a probability of 99%. In this study, a significant difference between 2 sets of samples means $P<0.01$.

## 4. Results

### a. Influence of the air percentage in the inlet gas

Continuous cultures were run under different levels of oxygen supply and the concentrations of all the measured metabolites during the different fermentations are shown in figure 1.

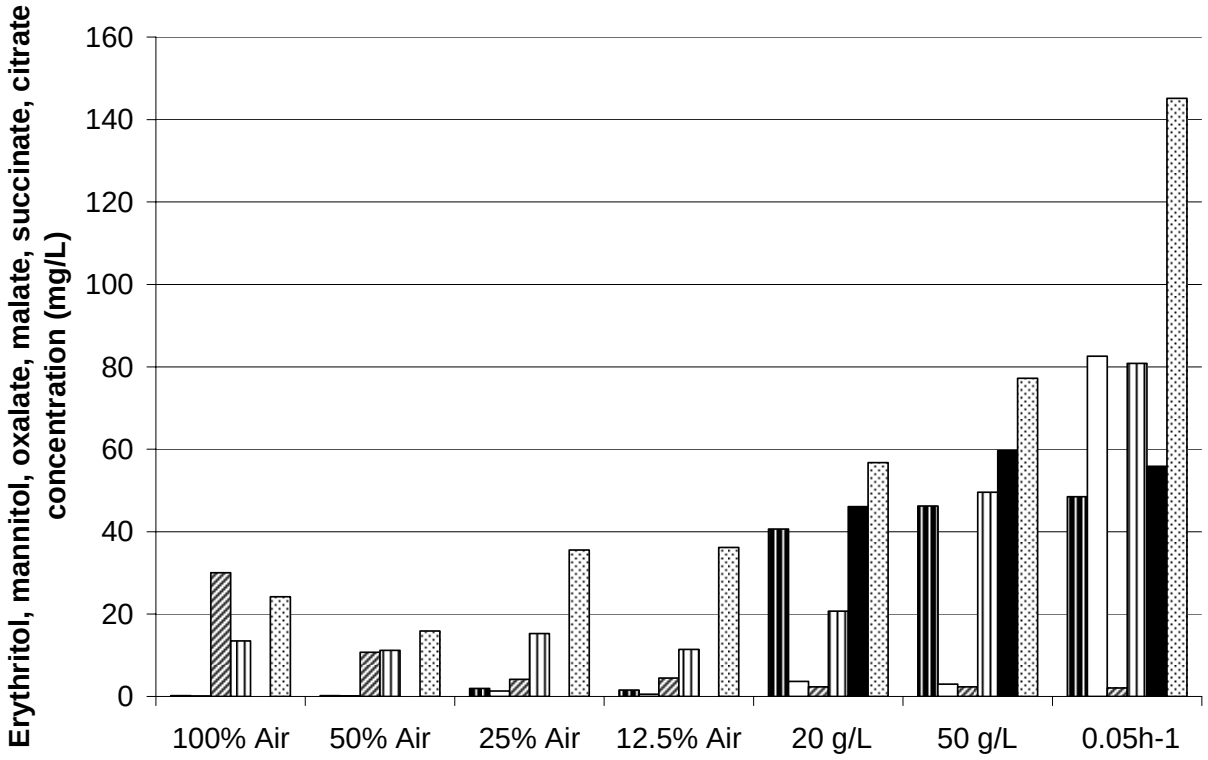


Figure 1 – Effect of oxygen availability, dilution rate and glucose concentration on the metabolite concentration: erythritol (▥), mannitol (□), oxalate (▨), malate (▤), succinate (■) and citrate (▩). The continuous cultivation conditions were: an air % of 12.5%, a glucose concentration of 8 g/L and a dilution rate of 0.1 $h^{-1}$, otherwise as indicated.

The different levels of oxygen supply were obtained by varying the mix of air/nitrogen in the inlet gas. The percentage of air in the mix varied from 100% to 12.5%. At 100% and 50%, no residual glucose was detected and the cultures were therefore considered to be carbon-limited. This was confirmed by the fact that the biomass concentration, the specific consumption rate of glucose ($r_{glu}$), the specific production rate of $CO_2$ ($r_{CO2}$) and the specific production rate of metabolites ($r_{met}$) were the same in these two fermentations (Table 1). The main metabolites produced were citric, oxalic and malic acids together with small amounts of pyruvic acid (Table 2).

Table 1 – Effect of oxygen availability, dilution rate and glucose concentration on the biomass concentration, concentration of glucose left in the medium, specific consumption rate of glucose ($r_{glu}$) and specific production rate of $CO_2$ ($r_{CO2}$) and metabolites ($r_{met}$). The average (Ave) and standard deviation (SD) were calculated on a minimum of 3 samples taken during the steady state.

| Air % | | 100 | 50 | 25 | 12.5 | 12.5 | 12.5 | 12.5 |
|---|---|---|---|---|---|---|---|---|
| Dilution rate ($h^{-1}$) | | 0.10 | 0.09 | 0.10 | 0.09 | 0.09 | 0.09 | 0.05 |
| $[glucose]_{feed}$ g/L | monohydrate glc | 8.00 | 8.00 | 8.00 | 8.00 | 20.00 | 50.00 | 8.00 |
| | HPLC measurement | 7.17 | 7.02 | 7.11 | 6.39 | 18.00 | 45.61 | 7.06 |
| [Biomass] g/L | Ave | **4.12** | **4.09** | **3.71** | **2.16** | **2.20** | **1.97** | **3.21** |
| | SD | 0.07 | 0.30 | 0.27 | 0.05 | 0.10 | 0.08 | 0.06 |
| [glucose]medium g/L | Ave | **0.00** | **0.01** | **0.57** | **2.84** | **14.17** | **42.06** | **0.98** |
| | SD | 0.00 | 0.00 | 0.08 | 0.10 | 0.37 | 0.18 | 0.08 |
| $r_{glu}$ $mmol.gDW^{-1}.h^{-1}$ | Ave | **0.95** | **0.95** | **0.94** | **0.86** | **0.91** | **0.96** | **0.48** |
| | SD | 0.00 | 0.00 | 0.01 | 0.03 | 0.09 | 0.05 | 0.01 |
| $r_{CO2}$ $mmol.gDW^{-1}.h^{-1}$ | Ave | **1.81** | **1.83** | **1.44** | **1.18** | **1.06** | **1.02** | **0.59** |
| | SD | 0.17 | 0.03 | 0.02 | 0.02 | 0.05 | 0.05 | 0.03 |
| $r_{met}$ $\mu mol.gDW^{-1}.h^{-1}$ | Ave | **14.43** | **7.98** | **11.35** | **17.37** | **62.17** | **98.33** | **44.83** |
| | SD | 1.96 | 0.36 | 1.11 | 1.35 | 6.03 | 3.90 | 2.12 |

Table 2 – Effect of oxygen availability, dilution rate and glucose concentration on the metabolite yields in $Cmmol.Cmol^{-1}$ glucose. The average and standard deviation were calculated on a minimum of 3 samples taken during the steady state.

| Air % | | 100 | 50 | 25 | 12.5 | 12.5 | 12.5 | 12.5 |
|---|---|---|---|---|---|---|---|---|
| D | $h^{-1}$ | 0.096 | 0.095 | 0.096 | 0.094 | 0.094 | 0.090 | 0.047 |
| [glucose]feed | g/L | 8.00 | 8.00 | 8.00 | 8.00 | 20.00 | 50.00 | 8.00 |
| Yglycerol | **Ave** | **0.00** | **0.02** | **0.06** | **0.10** | **3.18** | **4.91** | **1.15** |
| | SD | 0.00 | 0.00 | 0.01 | 0.00 | 0.64 | 0.18 | 0.09 |
| Yerythritol | **Ave** | **0.03** | **0.03** | **0.29** | **0.44** | **10.43** | **12.80** | **7.84** |
| | SD | 0.00 | 0.00 | 0.02 | 0.02 | 0.53 | 0.53 | 0.55 |
| Yarabitol | **Ave** | **0.01** | **0.01** | **0.05** | **0.10** | **1.01** | **0.96** | **1.66** |
| | SD | 0.00 | 0.00 | 0.00 | 0.01 | 0.13 | 0.05 | 0.06 |
| Ymannitol | **Ave** | **0.01** | **0.01** | **0.20** | **0.15** | **0.95** | **1.00** | **13.43** |
| | SD | 0.00 | 0.01 | 0.01 | 0.02 | 0.10 | 0.00 | 0.32 |
| Yoxalic acid | **Ave** | **2.86** | **1.04** | **0.44** | **0.86** | **0.42** | **0.45** | **0.23** |
| | SD | 0.50 | 0.01 | 0.01 | 0.17 | 0.05 | 0.03 | 0.04 |
| Ypyruvic acid | **Ave** | **0.34** | **0.40** | **0.27** | **0.92** | **1.10** | **2.24** | **2.77** |
| | SD | 0.00 | 0.04 | 0.02 | 0.42 | 0.35 | 0.02 | 0.22 |
| Ymalic acid | **Ave** | **1.72** | **1.46** | **2.14** | **2.94** | **4.96** | **12.80** | **12.18** |
| | SD | 0.00 | 0.00 | 0.00 | 0.00 | 1.35 | 0.90 | 0.55 |
| Yfumaric acid | **Ave** | **0.00** | **0.00** | **0.73** | **0.31** | **0.35** | **0.52** | **1.23** |
| | SD | 0.00 | 0.00 | 0.10 | 0.02 | 0.04 | 0.03 | 0.04 |
| Ysuccinic acid | **Ave** | **0.00** | **0.00** | **0.00** | **0.00** | **12.43** | **17.37** | **9.51** |
| | SD | 0.00 | 0.00 | 0.00 | 0.00 | 0.50 | 0.84 | 0.51 |
| Ycitric acid | **Ave** | **3.23** | **2.17** | **5.20** | **9.75** | **14.18** | **20.83** | **22.86** |
| | SD | 0.38 | 0.24 | 0.29 | 0.39 | 0.64 | 0.32 | 0.79 |

When the air percentage was decreased to 25% or 12.5% the residual concentration of glucose increased up to 2.80 g/L, and these continuous cultures were therefore considered to be oxygen-limited. In these cultures, there was a lower biomass concentration, and $r_{glu}$, and $r_{CO2}$ decreased, while $r_{met}$ increased. The higher $r_{met}$ for the chemostat sparged with 100% air is explained by a high production of oxalic acid under this condition. When the air percentage was decreased from 25% to 12.5%, the biomass concentration, $r_{glu}$ and $r_{CO2}$ decreased by a factor of 1.7, 1.1 and 1.2, respectively, while $r_{met}$ increased by a factor of 1.5.

Moreover, a decrease in the oxygen supply influenced the carbon fluxes, first by changes in the fluxes towards organic acids, i.e. the flux towards oxalic acid decreased when the oxygen availability decreased, while the flux towards the other organic acids such as malic, citric and pyruvic acids increased (Table 2). When the air supply was reduced from 25% to 12.5%, the pyruvic acid production increased by a factor of 3.5, whereas the production of citric acid and malic acid increased by a factor of 1.9 and 1.4, respectively.

The fluxes towards the polyols also increased but the polyol yield did not exceed 0.9 Cmmol polyol per Cmol glucose consumed. The main increase was found for erythritol and mannitol with yields of 0.44 and 0.15 Cmmol/Cmol, respectively, at 12.5% of air in the gas inlet.

The intracellular concentration of polyols was increased when the air percentage decreased, especially concerning erythritol, arabitol and mannitol, but their intracellular concentrations did not exceed 1.4 mg/g biomass at 12.5% air (Table 3).

Table 3 – Effect of oxygen availability, dilution rate and glucose concentration on the intracellular polyol concentration in mg/g biomass. The average and standard deviation were calculated on a minimum of two independent samples, otherwise indicated.

| Air % | | 50 | 25 | 12.5 | 12.5 | 12.5 |
|---|---|---|---|---|---|---|
| Dilution rate $h^{-1}$ | | 0.09 | 0.10 | 0.09 | 0.09 | 0.05 |
| [glucose]feed g/L | | 8.00 | 8.00 | 8.00 | 20.00 | 8.00 |
| Glycerol | Ave | **0.15** | **0.10** | **0.27** | **5.06** | **3.57** |
| | SD | 0.04 | 0.08 | 0.04 | n.d. | 0.45 |
| Erythritol | Ave | **0.14** | **0.58** | **1.39** | **16.25** | **5.87** |
| | SD | 0.01 | 0.07 | 0.07 | 4.93 | 0.31 |
| Arabitol | Ave | **0.53** | **0.50** | **1.38** | **5.24** | **3.23** |
| | SD | 0.01 | 0.44 | 0.44 | 1.38 | 0.02 |
| Mannitol | Ave | **0.76** | **1.53** | **1.39** | **10.54** | **23.61** |
| | SD | 0.04 | 0.17 | 0.14 | 3.91 | 0.88 |

It is interesting to notice that the morphology of mycelia was not significantly changed when the supply of oxygen was decreased. From 100% to 12.5% of air, the diameter of

the hyphae varied from 3.34 μm ± 0.55 to 3.58 μm ± 0.51, while the hyphal growth unit length was 33.18 ± 7.52 at 100% of air and 32.10 ± 7.36 at 12.5% of air.

### b. Influence of the dilution rate

A decrease of the dilution rate from 0.10 to 0.05 $h^{-1}$ with low oxygen supply had many consequences. As the limiting compound is oxygen, the decrease in the dilution rate led to an increase in the biomass concentration from 2.16 to 3.28 g/L and a decrease in the residual glucose concentration from 2.80 to 0.98 g/L. The consequences are a decrease in $r_{glu}$ by a factor of 1.8 followed by a decrease in $r_{CO2}$ by a factor of 2.0, whereas $r_{met}$ increased by a factor of 2.6 (Table 1). The increase of $r_{met}$ is due to an increase in the flux towards all the organic acids and polyols, with the exception of oxalate (Table 2). The yield of citric acid is increased by a factor of 2.3, the yield of pyruvic acid by a factor of 3.0 and the yields of fumaric and malic acids are increased by a factor of 4.1. Succinic acid, which was not produced at a dilution rate of 0.10 $h^{-1}$, was produced, at the low dilution rate, at a yield of 9.51 Cmmol/Cmol of glucose. The yields and intracellular concentrations of all polyols increased when the dilution rate was decreased, but the largest increase was observed for mannitol production. A 2-fold decrease in the dilution rate increased the mannitol yield 88 times, while its intracellular concentration increased 17 times. The hyphal diameter at 0.05 $h^{-1}$ was 3.13 μm ± 0.43 and was significantly different from the hyphal diameter at 0.10 $h^{-1}$.

### c. Influence of the glucose concentration in the feed

A variation in the glucose concentration in the feed from 8 to 50 g/L had consequences mainly on $r_{CO2}$ and $r_{met}$. Table 1 shows that $r_{CO2}$ decreased by a factor of 1.1 while $r_{met}$ increased by a factor of 5.7. This is linked to an increase in the flux towards all the organic acids and polyols, except for oxalic acid (Table 2). In particular the fluxes towards pyruvic, malic, citric and succinic acids were increased – succinic acid was not produced at 8 g/L whereas the yield was as high as 17.4 Cmmol/Cmol glucose at 50 g/L of glucose. Concerning the polyols, the main consequences of an increase in the glucose feed concentration were an increase in the production of erythritol and glycerol, which were successively excreted, while mannitol and arabitol were accumulated intracellularly (Table 3).

Moreover, an increase in the glucose concentration increased not only the carbon source concentration in the medium, but also the osmotic pressure imposed by the medium on the cells. To differentiate these two effects, glucose-limited chemostats were run at 17 mM, 0.5 M and 1 M NaCl. Interestingly, no real steady state could be reached for the chemostat using 1 M NaCl: the biomass, glucose and metabolite concentrations

were stable except for the glycerol and erythritol concentrations, which were fluctuating all along the fermentation, explaining the large standard deviation for these two compounds (Table 4). The increase in the NaCl concentration led to an increase in the extracellular concentration of glycerol and erythritol even though carbon was the limiting compound. From 17 mM to 1 M NaCl, the extracellular concentration of erythritol and glycerol increased respectively 10 and 16 times, while the intracellular concentrations increased 119 and 90 times, respectively (Table 4). Besides these two polyols, the other metabolites were not affected by the increase in the osmotic pressure.

Table 4 – Influence of osmotic pressure on the extracellular and intracellular concentration of polyols in mg/g biomass. Average and standard deviation were calculated on a mimimum of two independent sampling and extraction.

| | 17 mM NaCl | | | | 0.5 M NaCl | | | | 1 M NaCl | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | extracellular | | intracellular | | extracellular | | intracellular | | extracellular | | intracellular | |
| | Ave | SD | Ave | SD | Ave | SD | Ave | SD | Ave | SD | Ave | SD |
| glycerol | **0.03** | 0.00 | **0.15** | 0.04 | **0.32** | 0.01 | n.d. | n.d. | **0.51** | 0.29 | **13.09** | 4.47 |
| erythritol | **0.05** | 0.00 | **0.14** | 0.01 | **0.24** | 0.02 | n.d. | n.d. | **0.44** | 0.23 | **16.93** | 5.78 |
| mannitol | **0.02** | 0.01 | **0.76** | 0.04 | **0.02** | 0.00 | n.d. | n.d. | **0.04** | 0.00 | **0.56** | 0.09 |

### d. Oxygen step down experiments

Figure 2 shows the results from an oxygen-limited continuous culture, which was first sparged with 12.5% of air until a steady state was reached. After 172 h of cultivations, the percentage of air was suddenly decreased to 6.25%. The main consequences of this perturbation were a decrease in the biomass concentration, followed by an increase in the glucose concentration. The concentration of all metabolites decreased progressively except for the mannitol concentration, which increased to 119.1 mg/L. When the air supply decreased to 3.125%, the same consequences were observed. Interestingly, in this experiment, the mannitol concentration reached 230.5 mg/L, which is nearly the double of what is observed when the air percentage is decreased to 6.25%.

$r_{glu}$ was calculated for the last 20 hours of the cultivation sparged with 6.25% of air, and was estimated at 0.29 mmol/gDW/h. This emphasizes a decrease of the glucose consumption after perturbation of the steady state, as $r_{glu}$ was equal to 0.48 mmol/gDW/h in the first part of the cultivation. However, no real steady state was obtained after the perturbation: indeed such experiments increase the pressure of selection. It is interesting that ethanol, which is normally not produced by *A. niger BO1*, was detected at the end of the cultivations, which indicates a selection for cells that are better adapted to oxygen limitation.

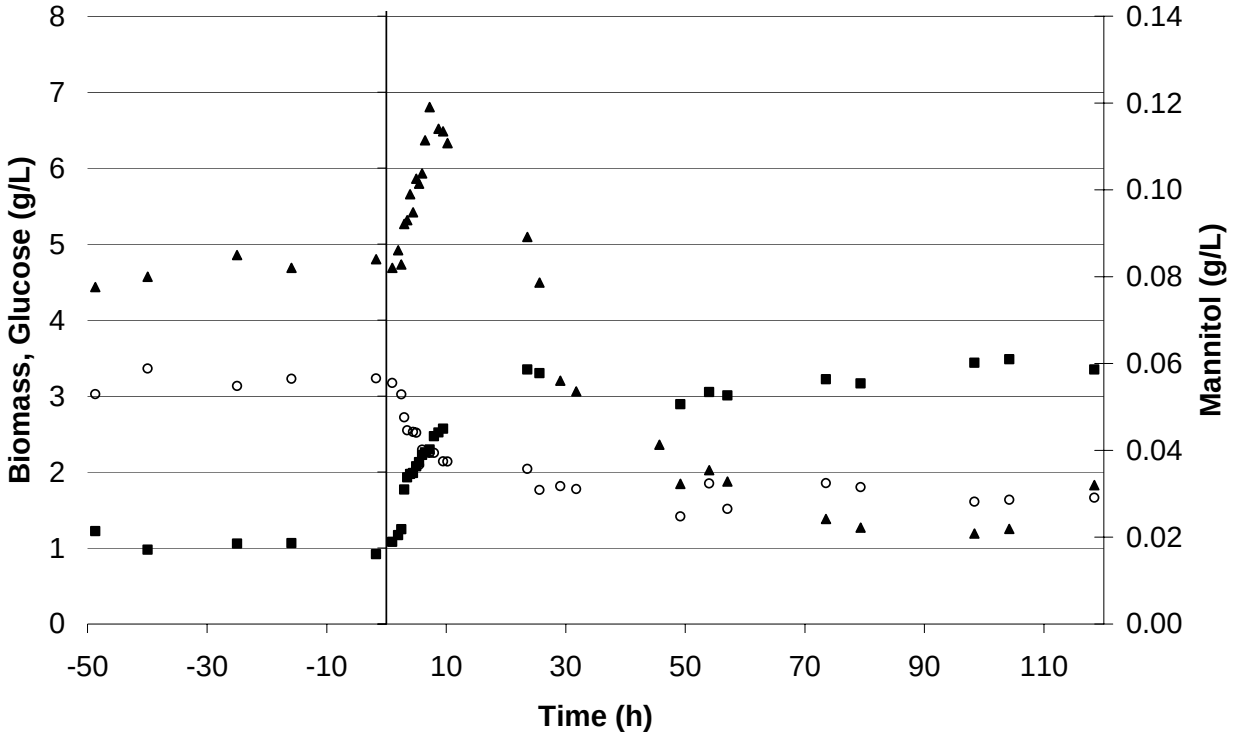


Figure 2 – Effect of a sudden decrease of oxygen availability on the biomass (○), glucose (■) and mannitol (▲) concentration. At t=0 the air % of the gas sparged into the bioreactor was decreased from 12.5 to 6.25%.

## 5. Discussion

### a. Influence of the air percentage in the inlet gas

When the percentage of air in the gas inlet was decreased from 50% to 25%, the compound limiting growth shifted from carbon to oxygen. Hereby not all glucose was consumed and the biomass concentration decreased. Moreover going from carbon limitation to oxygen limitation implies a shift in the metabolism. During carbon-limited cultures, very few metabolites are excreted. However, when a culture is oxygen-limited, the low availability of the final electron acceptor affects the reoxidation of NADH and hereby the production of ATP. This results in an imbalance in production and consumption of energetic cofactors, and leads to production of metabolites. In this study, a decrease in the oxygen supply resulted mainly in an increase in the production of organic acids. The organic acids produced were pyruvic, citric and malic acids, indicating that there was a limitation in the TCA cycle activity. The main role of the TCA cycle is to produce energetic cofactors NADH, $FADH_2$ and ATP as well as compounds involved in biomass synthesis. At oxygen limitation, these two functions can not be balanced, as the catabolic reduction charge is high due to an accumulation of NADH and $FADH_2$, resulting in an altered activity of the TCA cycle. In the particular case of the yeast *Saccharomyces*

*cerevisiae*, under anaerobic growth conditions, the TCA cycle is operating in a branched fashion and can hereby fulfill the biosynthetic demands, while limiting the problem with accumulation of NADH and $FADH_2$ (Gombert et al., 2001; Maaheimo et al., 2001).
Furthermore, Legisa and Grdadolnik (2002) have shown more generally that a decrease in oxygen availability leads to a decrease in the intracellular concentration of ATP, affecting the activity of ATPases. The transmembrane ATPases play an important role in the regulation of the cytoplasmic homeostasis and a decrease of their activity leads to a decrease in the intracellular pH (pHi) (Hesse et al., 2002; Legisa and Grdadolnik, 2002). A decrease in pHi has several effects. First, it results in a decrease in the activity of the NADP-dependent isocitric dehydrogenase, which could in turn lead to an increase in citric acid production (Mattey and Bowes, 1978). More generally, Jernejc and Legisa (2004), have shown that low pHi leads to an increase in the citric acid excretion, which would be a way for the cell to overcome low pHi. The study has been done only on citric acid but an increase in the excretion of other organic acids could theoretically also help reestablishing an optimal pHi. Moreover, Prömper et al. (1993) have shown that a deletion of the proton pumping respiratory chain, causing a decrease in ATP production, leads to an intracellular accumulation of TCA intermediates, up to 20-fold compared to the normal level. These observations may explain the accumulation of organic acids, such as pyruvic, citric and malic acids, observed in this study at low oxygen availability.
However, the increase in citric acid production when the oxygen availability decreases, contradicts the results of Khan and Ghose (1973) and Kubicek et al. (1980) who showed that high aeration levels are necessary for citric acid production and that low oxygen levels can irreversibly stop the production of this compound. First, one should notice that the cultivation conditions in the present work are different from the cultivation conditions described in the studies of Khan and Ghose (1973) and Kubicek et al. (1980), leading here to the production of free dispersed hyphae, while citric acid production is usually studied using a different medium and a different morphology (pellet), which are required conditions to enhance the production of this acid. One explanation for the differences observed could be linked to the activity of the respiratory chain enzymes. During citric acid production, using pellet growth, the activity of the proton-pumping NADH: ubiquinone oxidoreductase, also called complex I, decreases in favor of the alternative respiratory enzymes (Wallrath et al., 1991). Under these conditions, ATP is mainly produced via the glycolysis. Kubicek et al. (1980) have shown that the use of salicylhydroxamic acid (SHAM), which is an inhibitor of the alternative respiratory enzymes, strongly inhibits the production of citric acid without significantly affecting the biomass production. This implies that the use of SHAM shifts

the reoxidation of NADH from the alternative respiratory chain to the proton pumping respiratory chain. A similar observation has been made when the aeration was interrupted for up to 20 minutes. In conclusion, the results of these studies suggest that, in the course of citric acid production, a lack of oxygen shifts the reoxidation of NADH from the alternative respiration to the proton pumping respiration, leading to an increase in ATP production. As explained earlier, an increase of ATP leads to an increase of pHi and a decrease of organic acid production. In continuous cultures, where *A. niger* is grown as free dispersed hyphae, leading to the production of low amounts of citric acid, the scenario is different: the availability of oxygen seems to be linked to the intracellular concentration of ATP. Hereby there is a high probability that the main NADH reoxidation pathway is, in these conditions, the proton-pumping respiratory chain. In this case, a decreasing oxygen availability results in a decrease of the activity of the respiratory chain and therefore of the ATP production, which favors the citric acid production.

In the present study, the only acid having a lower production in oxygen limitation is oxalic acid. This could be explained by the fact that in *A. niger*, the only enzyme leading to oxalate, oxaloacetate acetylhydrolase (OAH), is under some conditions, highly sensitive to a decrease in pHi. Ruijter et al. (1999), using an imidazole buffer, observed a decrease of 50% of the OAH activity in vitro when the pH decreases from 7.5 to 6.5.

A decrease in pHi also affects considerably the activity of the hexokinase and the 6-phosphofructokinase (Haq et al., 2005). The consequence is a decrease in the Embden-Meyerhof-Parnas (EMP) pathway activity, which could explain the decrease in $r_{glu}$ when the availability of oxygen decreases.

The decrease in $r_{CO2}$ is also a marker of the metabolism shift, when the limitation shifts from carbon to oxygen. This decrease indicates a decrease in the activity of the TCA cycle and/or the PP pathway, which are the $CO_2$ producing pathways. This could be linked to the decrease of pHi at oxygen limitation which acts both on the activity of the NADP-dependent isocitrate dehydrogenase from the TCA cycle (Mattey and Bowes, 1978) and on the activity of the glucose-6-phosphate dehydrogenase from the PP pathway (Haq et al., 2005).

In a previous study, the influence of an oxygen limitation period has been studied in *A. niger BO1* in batch cultivation (Diano et al., 2006). It has been shown that low oxygen availability enhances the production of polyols and especially mannitol, as well as the production of succinate. In the present study, the specific production rate of mannitol did not exceed 0.20 Cmmol per Cmol glucose and succinate was not detected, while in the batch study mannitol and succinate represents respectively up to 88.55 and 23.6 Cmmol per Cmol of glucose. These differences observed for the same strain and under similar growth conditions may be due to the specific growth rate and carbon source

concentration differing in the batch cultivations and the chemostat cultures: indeed a period of oxygen limitation in the batch culture is associated with a low specific growth rate, while the carbon source concentration was up to 40 g/L during oxygen limitation.
Finally, the present study shows that oxygen availability does not influence the morphology of *A. niger*, which is in accordance with the study of Olsvik et al. (1993). The results of previous studies on *Aspergillus oryzae* (Rahardjo et al., 2005; Wongwicharn et al., 1999), *Aspergillus nidulans* (Carter and Bull, 1971) and *Penicillium chrysogenum* (van Suijdam and Metz, 1981) suggest that the effect of oxygen availability on the morphology of the hyphae is strain-dependent.

### b. Influence of the dilution rate

When the specific growth rate decreases, the requirements for biomass synthesis decrease, causing a reduction in the consumption of substrates, e.g. glucose and oxygen. Under oxygen-limited conditions, this leads to an increase in biomass concentration. In parallel, the yield of metabolites increases almost 5-fold, which leads to an increase in $r_{met.}$ This is explained by a positive effect of low specific growth rate on excretion of organic acids, a phenomenon which has previously been observed in ammonium-limited continuous cultures (Gallmetzer and Burgstaller, 2002). Furthermore, low specific growth rates also increases the production rate of polyols. Using carbon-limited chemostat, Ilyes et al. (2004) have shown that low specific growth rates lead to a derepression of the carbon catabolite repression. In *Aspergillus spp.*, carbon catabolite repression is mediated by CreA. In presence of D-glucose or other rapidly metabolised carbon sources, CreA inhibits the transcription of specific target genes involved in the catabolism of less preferred carbon sources (Felenbok and Kelly, 1996; Mogensen et al., 2006; Ruijter and Visser, 1997). CreA is thereby involved in a network of reactions and its deletion has several effects both on fungal morphology (Dowzer and Kelly, 1991; Ruijter et al., 1997b; Shroff et al., 1997) and physiology (David et al., 2005; van der Veen et al., 1995). Particularly, in *A. nidulans*, a deletion of *creA* results in an 11-fold increase of erythritol yield and in 2-fold increase of mannitol yield (David et al., 2005). In the present study, a decrease of dilution rate to 0.05 $h^{-1}$ results in an 18-fold increase of erythritol yield, while the yield of mannitol was increased 88 times. The large difference in mannitol production may be linked to the conditions of cultivation: fully aerobic in the *A. nidulans* experiments and under oxygen limitation in the present study. The manner in which carbon catabolite repression controls the production of polyols is still not clear. Van der Veen et al. (1995) suggested that the increase in polyol production is linked to the increase of the activity of hexokinase, associated with a decrease in the activity of phosphofructokinase, leading in turn to an overflow via pathways branching off from the glycolysis. These pathways could be mannitol and PP pathways, which is in

line with our study, where the intracellular and extracellular concentrations of mannitol, arabitol and erythritol increased at low dilution rates. However, it is important to notice that, in the study of Van der Veen, an increase in the fructose-2,6-bisphosphate concentration is observed in the c*reA* mutant, which is believed to activate the phosphofructokinase (Ruijter et al., 1997a). The *in vitro* activity measurements may therefore be different from the *in vivo* values. However the authors have measured, in a *creA* mutant, an increase in the activity of MPD associated with an increase in the mannitol production and this could suggest a control of the MPD expression by the carbon catabolite repression. This hypothesis has been rejected by Singh et al. (1988) and by Ruijter et al. (2003). Singh et al. have measured the activity of enzymes involved in the mannitol pathways both during growth on a carbon catabolite repressing source and a carbon catabolite derepressing source. They did not find any significant differences in the enzyme activities between the two conditions. Ruijter et al. have measured the expression of MPD in a wild type strain of *A. niger* and in a *creA* mutant and they did not find any significant differences, although a CreA binding site has been found in the promoter of the MPD encoding gene. These observations may be explained by two possibilities: first, CreA is indeed not involved in mannitol production and the observations made by David et al. (2005) and Van der Veen et al. (1995) are more likely linked to the decrease in the specific growth rate observed for the *creA* mutant than to the carbon catabolite repression itself – or, second, mannitol production is effectively under the control of carbon catabolite repression, but also under the control of oxygen availability. Indeed, we showed previously that mannitol is excreted only during oxygen limitation (Diano et al. 2006) and studies in *S. cerevisiae* have shown the importance of oxygen availability both on the expression of several genes and on the activity of specific proteins (Burke et al., 1997; Castello et al., 2006; Kwast et al., 1999). It is also important to notice that the *A. niger creA* mutant used by Ruijter et al. (1993) was obtained by UV mutagenesis and selected depending on its morphology and physiology (Ruijter et al., 1997b). The mutagenesis using UV creates point mutations which affect the protein functions but does not ensure a completely non-functional protein. Such point mutations have been studied in the particular case of *A. nidulans creA* gene and the authors concluded that it is unlikely that any of the mutations characterized completely abolishes the binding of CreA on all promoters under its control (Shroff et al., 1996).

### c. Influence of the glucose concentration in the feed

The main step controlling the EMP pathway activity at low glucose concentration is the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (Karaffa and Kubicek, 2003; Peksel et al., 2002). An increase in the concentration of glucose increases the activity of the 6-phospho-2-fructokinase (Kubicek-Pranz et al., 1990; Torres et al.,

1996). This triggers the intracellular concentration of fructose-2,6-bisphosphate, which in turn activates the 6-phosphofructokinase (Ruijter et al., 1997a). Therefore, an increase in the glucose concentration in the feed increases the flux trough the EMP pathway, which leads to an increase in $r_{glu}$. As the production of biomass is limited by the oxygen availability, an increase in $r_{glu}$ leads to an overflow of metabolites such as citric, succinic and pyruvic acids. The positive effect of extracellular glucose concentration on the excretion of TCA cycle intermediates has been reported in many studies (Legisa and Kidric, 1989; Papagianni et al., 1999; Peksel et al., 2002; Torres et al., 1996; Xu et al., 1989). Citric acid is often excreted in high amounts, and Gallmetzer and Burgstaller (2002) have named this phenomenon an "energy spilling process" as citrate excretion limits the production of NADH.

An increase in glucose concentration also increases the production of polyols, particularly erythritol and glycerol. The results from the glucose-limited chemostats with different concentrations of NaCl indicate that the overproduction of erythritol and glycerol was linked to an increase in the osmotic pressure. Indeed, high levels of glucose increase the osmotic pressure of the medium, which leads to an intracellular accumulation of glycerol and erythritol in order to maintain an osmotic balance. However, the efficiency of this system is affected by the leakage of polyols through the membrane, as glycerol and erythritol can be detected in the medium. Moreover, in batch cultivation, high concentrations of carbon source lead to high levels of extracellular glycerol and erythritol (Diano et al. 2006). One should also notice that the intracellular concentration of erythritol in the continuous culture, using 1 M NaCl and having an osmotic pressure above 2 osmol/L, is equal to the intracellular concentration in the continuous culture using 20 g/L of glucose, where the osmotic pressure of the medium did not exceed 0.2 osmol/L. This shows a double effect of the osmotic pressure and carbon concentration on the production of erythritol.

The decrease in $CO_2$ production when the concentration of glucose increases may be linked to an increasing activity of the pyruvate carboxylase (Hossain et al., 1984).

### d. Oxygen step down experiments

A sudden decrease in oxygen availability puts the cell under high stress. The growth rate decreases and a part of the biomass is washed out until a new equilibrium between oxygen requirements and oxygen availability is reached. During this period of extreme oxygen limitation, the specific production rate of mannitol is doubled, underlying the importance of mannitol production and especially MPD under oxygen limitation. The fact that mannitol production increased almost twice when the percentage of air was decreased by a factor of 2 shows the tight control exerted by oxygen on the mannitol

pathway. Hypothetically, this control could occur through the intracellular level of NADH.

These experiments, using a sudden decrease in oxygen availability, are a powerful tool to study, in a controlled fashion, the metabolic response occurring in batch cultivation during the shift from exponential growth phase to oxygen limitation. Indeed, in oxygen-limited continuous cultures, although the growth is limited by the oxygen availability, a balance exists between the cellular requirement for oxygen and the oxygen supply. In batch cultivations, when the dissolved oxygen is depleted from the medium, the cells present in the bioreactor have to deal with the oxygen available, which is given by the oxygen transfer from the gas to the liquid phase. At this time, there is an unbalance between the oxygen requirements and the oxygen availability. The step down in oxygen level mimics this transient. This phenomenon explains also the difference in yields observed between the oxygen-limited continuous cultures and the batch experiments.

## 6. Conclusion

A limitation of oxygen is likely to result in an increase in the NADH/NAD ratio and to lead to a decrease in ATP production causing a decrease in pHi. These phenomena may affect the activity of several enzymes as well as the carbon flux distribution, leading to an accumulation of organic acids and especially citric acid. Moreover, in batch cultivation, oxygen limitation is associated with low specific growth rates. The present study shows that low specific growth rates increase the production of organic acids and polyols, which may be linked to a relief of the carbon catabolite repression under these conditions. The pulse experiments underline that the main consequence of extreme oxygen limitation is the production of mannitol. These experiments confirm the role of MPD as the main enzyme helping the reoxidation of NADH when the final electron acceptor, oxygen is limiting. In continuous cultures and batch cultivations, the production of mannitol under oxygen-limited conditions is associated both with low levels of oxygen and low specific growth rates. The chemostats at different NaCl concentrations show that glycerol production is linked to an increase in the osmotic pressure, while erythritol production is linked both to high osmotic pressure and to high concentrations of carbon.

Finally, this work underlines the advantages of using continuous cultures versus batch cultivations. The present study has been able to give details on the function and the regulation of polyols, which has been difficult to obtain from batch cultivations, where different parameters like oxygen availability, specific growth rate, and the concentration of glucose vary simultaneously. The use of continuous cultures also provided new insight into the effect of oxygen limitation on the production of citric acid.

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# IV. Construction and characterisation of *Aspergillus niger* strains lacking dehydrogenase activity by deletion of the *mpdA* and *gfdA* genes.

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*The results of this chapter will form the basis for a publication (in preparation).*

## 1. Introduction

Industrial production of enzymes using the filamentous fungus *Aspergillus niger* generally involves high density fermentations resulting in the occurrence of oxygen limitation. As for many other fungi, oxygen limitation leads to the production of polyols (Diano et al., 2006). Under oxygen-limited conditions, NADH is accumulated, but through the production of polyols the cells activate non-oxygen requiring pathways for oxidation of NADH and hereby allow intracellular balancing of the important co-factor NADH.
In *A. niger* the most important polyols secreted in high density fermentations are mannitol and glycerol, which can represent up to 19% of the carbon consumed (Diano et al., 2006). The production of mannitol involves MPD. MPD oxidises NADH to NAD by converting fructose-6-phosphate (F6P) into mannitol-1-phosphate (M1P). M1P is then dephosphorylated into mannitol (Figure 1).

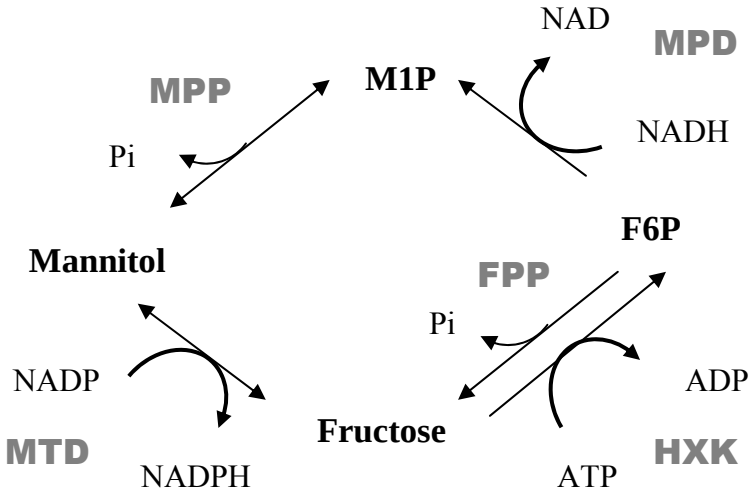


Figure 1 - Mannitol metabolism in *Aspergillus spp.* F6P, fructose-6-phosphate; M1P, mannitol-1-phosphate; HXK, hexokinase; MTD, mannitol dehydrogenase; MPP, mannitol-1-phosphate phosphatase; MPD, mannitol-1-phosphate dehydrogenase.

The production of glycerol involves the NAD-dependent glycerol-3-phosphate dehydrogenase (G3PDH) (Ansell et al., 1997; Costenoble et al., 2000). G3PDH converts dihydroxyacetone phosphate (DHAP) into glycerol-3-phosphate (G3P), while oxidising the cofactor NADH to NAD. G3P is subsequently dephosphorylated into glycerol. This pathway has been extensively studied in *Saccharomyces cerevisiae* where G3PDH is encoded by two genes, GPD1 and GPD2. GPD1 is mainly expressed in response to high osmotic pressure (Albertyn et al., 1994; Ansell et al., 1997), whereas GPD2 is expressed under

anaerobic or microaerobic conditions (Ansell et al., 1997; Costenoble et al., 2000; Eriksson et al., 1995).

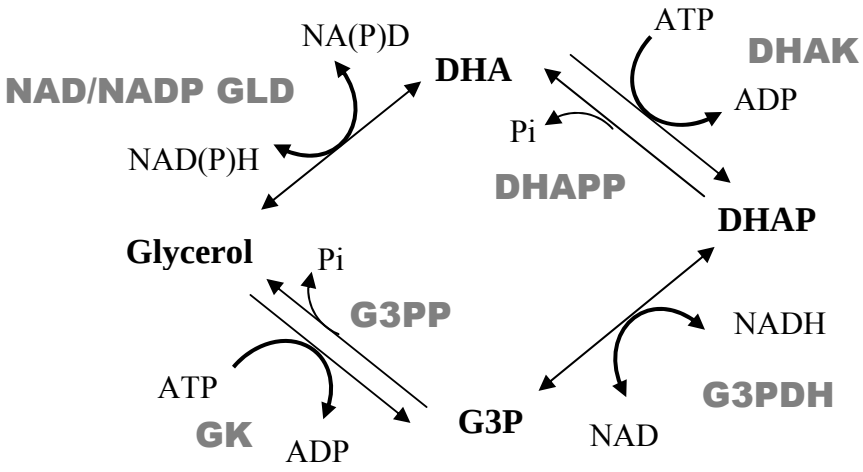


Figure 2 - Glycerol metabolism in Aspergillus spp. DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; G3P, glycerol-3-phosphate; G3PDH, glycerol-3-phosphate dehydrogenase; GK, glycerol kinase; G3PP, glycerol-3-phosphate phosphatase; GLD, NADP or NAD-dependent glycerol dehydrogenase; DHAK, dihydroxyacetone kinase; DHAPP, dihydroxyacetone phosphate phosphatase.

As mentioned earlier, the production of mannitol is the main pathway involved in NADH reoxidation during oxygen limitation in *A. niger* (Diano et al., submitted; Diano et al., 2006). The production of glycerol has been observed in oxygen-limited batch cultivations (Diano et al., 2006), but it was not possible to establish a direct link between oxygen limitation and glycerol production using oxygen-limited continuous cultures (Diano et al., submitted). This may be due to a successive production and reconsumption of glycerol. Indeed, in contrast to *S. cerevisiae*, glycerol anabolism and catabolism of *Aspergillus spp.* can proceed via 2 routes (Figure 2), which may lead to the presence of futile cycles (Schuurink et al., 1990). Moreover, the production of glycerol via one or the other route seems to depend on the growth conditions. For example, in response to high osmotic pressure, the activity of the route via DHA is increased, which leads to an accumulation of glycerol, while the activity of the route involving G3PDH is not affected (de Vries et al., 2003; Fillinger et al., 2001). The role of this last route, via G3P, has not yet been characterised and could be involved in oxygen limitation by analogy with the metabolism of *S. cerevisiae*. Similarly, even though the importance of the mannitol pathway has been underlined in oxygen limitation (Diano et al., submitted; Diano et al., 2006), as well as during germination (Witteveen and Visser, 1995) and sporulation (Chaturvedi et al., 1996; Ruijter et al., 2003), there is no study on its role under non-limited growth condition. The roles of both dehydrogenases, MPD and G3PDH, were therefore studied in both non-limited growth and oxygen-limited growth conditions. Mutants deleted for each of these genes were constructed, as well as a double deletion mu-

tant. The morphology and physiology of the mutants were analyzed in batch cultivations, during both the exponential growth phase and oxygen-limited growth phase.

## 2. Material and methods

### a. Strains

In this study, the *A. niger* strains used were derived from the industrial strains *A. niger BO1* and *A. niger Jroy3* (Pedersen et al., 2000). They are listed in table 1. All strains were maintained as frozen spore suspensions, at -80°C, in 20% glycerol.

Table 1 - *A. niger* strains used in this study

| Strain | Genotype | References |
|---|---|---|
| BO1 | *aglA* | Novozymes, Pedersen et al. (2000) |
| Jroy3 | *aglA pyrG* Δ | Novozymes |
| A10 | *aglA pyrG* Δ *mpdA* Δ::(*A. oryzae pyrG*) | This study |
| A8 | *aglA pyrG* Δ *mpdA* Δ::(repeat-*A. oryzae pyrG*-repeat) | This study |
| A8- | *aglA pyrG* Δ *mpdA* Δ::repeat | This study |
| A87 | *aglA pyrG* Δ *mpdA* Δ::repeat *gfdA* Δ::(*A. oryzae pyrG*) | This study |
| A7 | *aglA pyrG* Δ *gfdA* Δ::(*A. oryzae pyrG*) | This study |

### b. Localisation of genes

The *A. niger* genome sequences 4x and 8.9x coverage were used. These sequences were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). Localisation of the genes were carried out using the *A. niger mpdA* gene sequence (Ruijter et al., 2003) and the *A. nidulans mpdA* and *gfdA* sequences (Fillinger et al., 2001; Galagan et al., 2005) as well as the Blast tool of BioEdit (BioEdit v7.0.5: http://www.mbio.ncsu.edu/BioEdit/bioedit.htmL) (Altschul et al., 1997). The alignment score was calculated using a BLOSUM62 matrix. The gap penalties were 11 for the presence of a gap and 1 for extension. The Center for Biological Sequence (CBS) server was used for gene localisation (HMM: http://www.cbs.dtu.dk/services/HMMgene/, using *Candida elegans* as a model, (Krogh, 1997) and for prediction of splice sites (Netgene2: http://www.cbs.dtu.dk/services/NetGene2/, using *human sp.* as a model (Brunak et al., 1991; Hebsgaard et al., 1996). The gene database, NCBI, was used for the identification of known genes in a given DNA sequence (tblastx, http://www.ncbi.nlm.nih.gov/BLAST/)

### c. DNA manipulation

Standard methods were used for DNA manipulations such as PCR, Southern analysis and DNA digestion (Sambrook and Russel, 2001). The *Bst*EII-digested phage lambda

(cI857 *Sam* 7) DNA was used as DNA ladder in PCR and Southern blot analyses. PCR primers used are listed in table 2. Plasmids DNA were isolated using the Jet quick spin column technique (Genomed). PCR products were isolated using GFX PCR DNA and gel band purification kit (Amersham-Biosciences). The cloning was done using BD in-Fusion PCR cloning kit (BD Biosciences clontech), following the protocol PT3650-1. The plasmids used for cloning were derived from pCR4Blunt TOPO (invitrogen, Gaithersburg, MD) in which the gene *pyrG* from *A. oryzae* and the herpes simplex virus type-1 thymidine kinase gene were inserted. One vector, pDelR, contained the markor *pyrG* with repeats: 374 nucleotides present in duplicate upstream and downstream *pyrG* and another vector, pDel, contained *pyrG* without repeat. The thymidine kinase gene allows reducing end-joining insertion on 5-fluoro-2-deoxyuridine plate (Sachs et al., 1997). The deletion plasmid, pAudd10, was constructed by inserting in pDel a 1343 bp PCR fragment, amplified using the primers a and b, upstream *pyrG* and a 927 bp PCR fragment, amplified using the primers c and d, downstream *pyrG* (Figure 3). pAudd8 was constructed in the same way as pAudd10 using the vector pDelR as a template. pAudd7 was constructed by inserting a 1026 bp PCR fragment, amplified using the primers e and f upstream *pyrG* and a 2193 bp PCR fragment, amplified using the primers g and h, downstream *pyrG*.

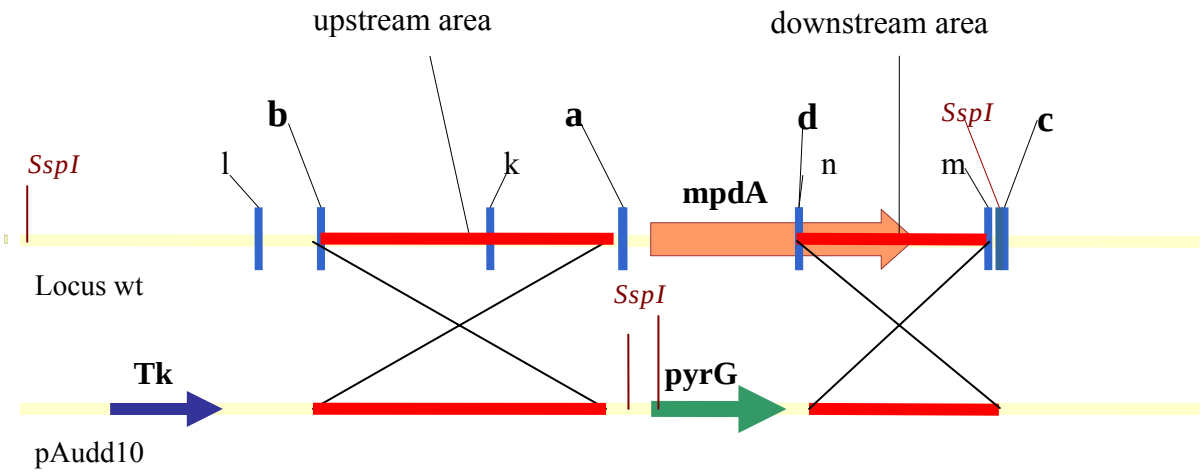


Figure 3 - Homologous recombination between *A. niger Jroy3 mpdA* locus and pAudd10. Bold letters indicate the location of primers used for the construction of pAudd10, while regular letters indicate the location of primers used for the Southern blot analysis. Restriction sites of *Ssp*I, used for Southern blot analysis, are also identified.

Chromosomal DNA was isolated using the MasterPure Yeast DNA purification (Epicentre Biotechnologies). Constructs were verified by sequence analysis. Transformations have been done using the linearised plasmids described in this paragraph (pAudd10, pAudd8 and pAudd7), following the method described by Nielsen et al. (2006) and using 4 µg of DNA per transformation. Transformants were isolated 3 times and checked by

Southern blot using downward capillary transfer (Sambrook and Russel, 2001). Southern analysis of A8 and A10 was performed on DNA digested by *Ssp*I using 2 probes: a 1039 bp PCR fragment (PCR primers k and l) and a 858 bp fragment (PCR primers m and n) (Appendix III). Southern blot of A87 was carried out using DNA digested by *Nco*I or *Kpn*I and 3 probes: a 1076 bp probe (PCR primers o and p), a 767bp fragment (PCR primers: q and r) and a 746 bp fragment (PCR primers s and t) (Appendix IV and V). A7 was analysed by Southern on DNA digested with *Nco*I or *Kpn*I using 1 probe of 1076 bp, constructed using the PCR primers o and p (Appendix IV).

Table 2 - Primers used during this study

| Primer sequence | Name |
|---|---|
| CGCGAAAGAGAAGAGTGGTG | a |
| CTCATACACTCGGACTTTG | b |
| GATTCAAGAGTGGTCGGAG | c |
| GCCATGCTACTACTGCCTAC | d |
| ACTAGACCTGACTAGGCCGAC | e |
| ACCTTCTTCGTTCACATCGAC | f |
| GTATGTCCGTTGGAGATATTC | g |
| GAATGGAAGATTGACCTCGTC | h |
| TGAGTGGTGCTACTGTAAG | k |
| CACTAACCACGTGCTAGTTG | l |
| AGAAGAGGAAGCCTGTGTG | m |
| CCATGCTACTACTGCCTAC | n |
| GTATGTCCGTTGGAGATATTC | o |
| GAAAGACTCGCCCTTCTC | p |
| TGAGTGGTGCTACTGTAAG | q |
| CTCATACACTCGGACTTTG | r |
| CAGTGAACAATTTGCGCTC | s |
| CATCCTTTCATACTCCTGATC | t |
| TCCCAGTCCGACTTCTTCTC | RT1_actin |
| TGCGTTCATCGTCCCTTG | RT2_actin |
| GCCTATGATCCTCCGCACAT*G* | RT3_gfdA |
| AAGAAGGAATATCTCCAACGGA*C* | RT4_gfdA |
| GATGTGGAGGAGCGTGATG | RT5_gfdB |
| CCTTCAACTGGTCGTGCTTC | RT6_gfdB |

### d. Reverse Transcriptase PCR (RT PCR)

Duplicate samples were taken for total RNA extraction, using sterile material pre-cooled at –20°C. Biomass was successively filtered through miracloth, dried using several sheets of disposable paper and put into liquid nitrogen. Samples were kept at –80°C until further analyses. Total RNA was extracted using RNAeasy kit (Qiagen) as explained by Mogensen et al. (2006) followed by a DNAse treatment using the DNAseI RNAse free enzyme (New England Biolabs). Reverse transcription using the M-MLV Reverse transcriptase RNase H Minus (Promega Corporation) has been done following the manufacturer instructions and a poly-dT primer. The second strand synthesis has been done by standard PCR techniques (Sambrook and Russel, 2001) using the primers poly dT and the forward primers RT1_actin, RT3_*gfdA* and RT5_*gfdB* (Table 2). The next PCR was carried out using the same forward primers and the respective reverse primers: RT2_actin, RT4_*gfdA* and RT6_*gfdB* (Table 2). The quality of these sets of primers has been controlled on DNA template. During RT PCR, actin has been used as a control gene (positive control). To check any DNA contamination in the RNA samples, RT PCR has been run as well on the RNA samples omitting the reverse transcriptase step (negative control).

### e. Cultivations

#### *Media*

The medium composition for submerged cultivations was the same as described previously (Diano et al., 2006) using 12 g/L of $NH_4Cl$ as ammonium source and 110 g/L of monohydrate glucose as carbon source.

Plate cultivations were done on minimal medium supplemented by a final concentration of 1% (w/v) carbon source, unless indicated otherwise. The influence of osmotic pressure was studied by adding 1 M NaCl.

#### *Cultivation conditions*

Shake flask cultivations were run using 100 mL medium with an initial spore concentration of $3.10^8$ spores/L. The shake flasks were incubated for 40 hours at 30°C and 230 rpm. Uridine was added to obtain a final concentration of 20 mM in the shake flasks.

For the batch cultivations, the fermentation set up and cultivation conditions were the same as described previously (Diano et al., 2006), except concerning the working volume: 4.5 L and the agitation: 700 rpm. Fermentations of *A. niger BO1, A10, A7* and *A87* were carried out in duplicate. For each fermentation, duplicate samples for biomass and

extracellular metabolites were taken every 3-4 hours. Average and standard deviation were calculated on these 4 sets of samples unless indicated otherwise.
Plates were inoculated in duplicate with spores harvested from a minimal medium plate containing 1% glucose, unless indicated otherwise. Colonies were grown for 4 days at 30°C.

***Metabolite quantification***

The quantification of substrate and products was carried out as described previously (Diano et al., 2006). Malic acid was quantified using the same HPLC set up as described previously (Diano et al., 2006) but using 2 mM $H_2SO_4$ as eluent. Trehalose, arabitol, xylitol, acetate and ethanol were measured, but data on these compounds will not be mentioned further, as very low amounts of trehalose and arabitol were measured (corresponding to less than 0.01 Cmol/Cmol glucose) and no xylitol, acetate nor ethanol were detected.

***Preparation of cell extract and enzyme assays***

Samples for enzyme assays were taken in duplicate, filtered through miracloth, washed with a 0°C sterile solution of NaCl 0.9%, dried using several sheets of disposable paper and frozen at -80°C until further analyses. The frozen samples were thawed on ice and, for each sample, 0.5 g was transferred to a 2-mL FastPrep tube containing 0.5 mL glass beads (diameter of 0.75 to 1 mm) together with 1mL extraction buffer (0.1 M sodium phosphate buffer at pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA and 2.6 mM dithiothreitol). The FastPrep tubes were processed 4 times 10s on a FastPrep FP120 Instrument (Savant Instruments, New York), with cooling on ice in between, at speed setting 5. After disruption, samples were centrifuged at 13,000 g at 4°C for 2 min and the supernatants were analysed for enzyme activity. Enzyme assays were performed in triplicate at room temperature using a microplate reader (Multiskan Ascent, Thermo Electron Corporation). The determination of G3PDH activity was done following the protocol SPDHAP02 from Sigma-Aldrich (http://www.sigmaaldrich.com). The same protocol was used for the measurement of MPD activity using 50 mM of F6P as substrate solution.

***Morphological analyses***

The analysis of the fungal morphology was realised as described by Haack et al. (2006). Samples were taken during germination, in the middle of the exponential phase and in the oxygen limitation phase, while a minimum of 5 g/L of glucose were still present in the medium. For each sample a minimum of 10 pictures were taken and from each picture the diameter of the hyphae in the subapical compartment were measured 3 to 4

times. In total, an average of 40 values was collected per sample, on which average and standard deviation were determined. Comparison between two strains or two conditions was carried out using a Student's t-test with a confidence level of 95%. In this study, a significant difference between 2 sets of samples means $P<0.05$ while similarity means $P>0.05$ (Livingston, 2004).

# 3. Results

## a. Identification of the genes encoding for MPD and G3PDH in *A. niger* genome

### *Gene encoding MPD*

The annotation of the *A. nidulans* genome predicted the existence of a single ORF, *mpdA* encoding MPD (David, 2005). Moreover, using a cDNA library, Ruijter et al. (2003) have identified the *A. niger mpdA* gene. *mpdA* is an intron-free Open Reading Frame (ORF) encoding a protein of 388 amino acids, having in the promoter domain a CreA binding site as well as a TATA like box. A homology search (tblastn) of *A. nidulans* and *A. niger* MPD sequences on the *A. niger* genome 8.9x coverage confirmed the existence of a single *A. niger mpdA* ORF localized on scaffold 2 at the position 1463843 to 1465009 (Figure 4).

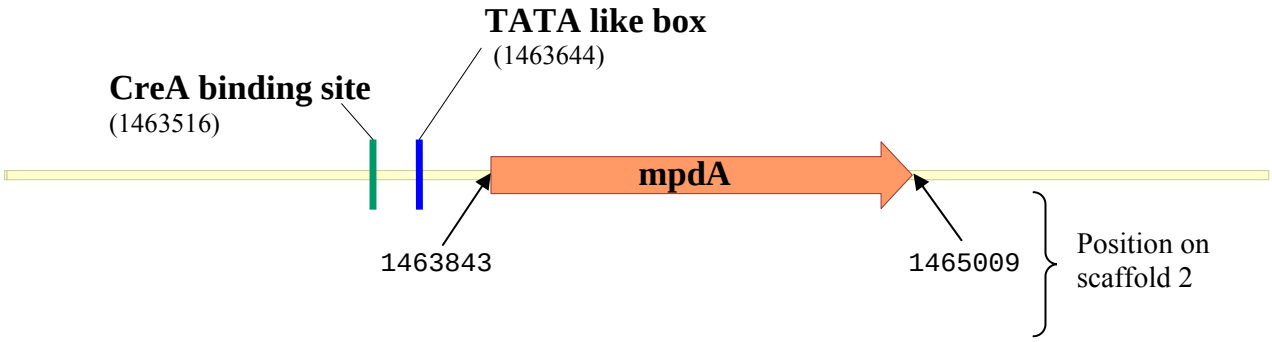


Figure 4 - Localisation and characterization of *mpdA* gene in *A. niger* genome sequence.

### *Genes encoding G3PDH*

*Localisation and characterisation of the genes*

No information on the *A. niger* G3PDH encoding gene(s) was available. However, the recently annotated genome of *A. nidulans* leads to the assignment of two ORFs, AN0351.2 and AN6792.2 corresponding to the *A. nidulans* G3PDH (David et al., 2006). The first ORF, AN0351.2, has been fully characterised by Fillinger et al. (2001). The amino acid sequences of both ORFs were used for a homology search (tblastn) on the *A. niger* genome sequence with 8.9x coverage. These blasts allowed the identification of

two potential ORFs encoding for *A. niger* G3PDH. The translated sequence of the first ORF, GfdA, has 82% identity with the *A. nidulans* GfdA amino acid sequence described by Fillinger et al. (2001), while the translated sequence of the second ORF, GfdB, has 69% identity with the amino acid sequence corresponding to AN6792.2 (Appendix I and II).

The gene *gfdA* is localised on the complementary strand of the scaffold 1 of the *A. niger* genome sequence, between the nucleotide 2176251 and 2177643. The existence of an ORF at this position has been confirmed by the gene finder program HMM. Moreover, the alignment presented in Appendix 1 suggests the presence of two introns of respectively 75 and 68 bp in the *A. niger gfdA* gene (Figure 5). A TATA-like box, TATAAG, was located 87 nucleotides upstream of the start codon. No potential CreA binding domain was identified within the 1000 nucleotides upstream the gene.


Figure 5 - Localisation and characterization of *gfdA* gene in *A. niger* genome sequence.

The second ORF, *gfdB*, was localised on the complementary strand of the scaffold 6 and includes the nucleotides 1864402 to 1865728. Using the nucleotides 1864000 to 186600 as a query sequence, the gene finder server, HMM, predicted a coding sequence starting at the position 1865740 and finishing at 1864413. The Netgene2 server on the same query sequence predicted the presence of two introns: one between 1865685-1865694 and 1865588-1865610 and a second one localised at the end of the gene between the nucleotides 1864516 and 1864458. To get more information, both sequences *gfdA* and *gfdB* were aligned with *gfdA* cDNA (Figure 6).

```
gfdA-A.niger            1 ATGGGCTCTCTCGGACCGTATCAGCGCAAACACAAGATCACTGTGGTGGGCTCAGGAAAC
gfdA-cDNA-A.niger       1 ATGGGCTCTCTCGGACCGTATCAGCGCAAACACAAGATCACTGTGGTGGGCTCAGGAAAC
gfdB-A.niger            1 -------ATGCATGAGCACAC--ACGAAAGCACAAGGTGGCCGTGGTCGGCTCCGGTAAT

gfdA-A.niger           61 TGGTATGTGCAAGCTGGCTTTCCGTCATGGTGCTAGTGCCAGGGAAGAAGGAAAAGGCTG
gfdA-cDNA-A.niger      61 TGG------------------------------------------------------------
gfdB-A.niger           52 TGGTATGCACCAACCCTTTCTTTCCGCTGCCGCGCATTT---------------GGCTA

gfdA-A.niger          121 ACTCGGGTCATGTATAGGGGATGCGCAATCGCCAAAATTGTCGCCGAAAATGCCGCCAGC
gfdA-cDNA-A.niger      64 ----------------GGATGCGCAATCGCCAAAATTGTCGCCGAAAATGCCGCCAGC
gfdB-A.niger           96 ATACCGTTGTAATCTAGGGGATCCACCATCTCCAAGATTGTTGCGGAGAACGCCAGAGAG
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gfdA-A.niger          181 AACCCCGCAATTTTCGAGGAGAAGGTTGAGATGTGGGTTTTTGAGGAGAAGGTGGAAATC
gfdA-cDNA-A.niger     106 AACCCCGCAATTTTCGAGGAGAAGGTTGAGATGTGGGTTTTTGAGGAGAAGGTGGAAATC
gfdB-A.niger          156 CATTCGGACCTTTTCGAGCCAGAAGTGCGCATGTGGGTGTTTGAGGAGGAAATCGAAATC

gfdA-A.niger          241 TCCAAAGACTCCCGGCACTATGATCCCTCTTCACCGCTGTGCCAGGGCCCACAAAACCTG
gfdA-cDNA-A.niger     166 TCCAAAGACTCCCGGCACTATGATCCCTCTTCACCGCTGTGCCAGGGCCCACAAAACCTG
gfdB-A.niger          216 CCTGAATCCTCCAAGCACCA------CTCCAAACTAGGCGGCCAGAA---GCACAAATTG

gfdA-A.niger          301 ACGGATGTGATCAACCAAAAGCACGAGAACATAAAATACCTGCCAGGAATTACTCTCCCC
gfdA-cDNA-A.niger     226 ACGGATGTGATCAACCAAAAGCACGAGAACATAAAATACCTGCCAGGAATTACTCTCCCC
gfdB-A.niger          267 ACCGAGGTCATCAACGGAGTCCACGAGAATGTCAAATACCTGCCGGATATTGCACTCCCT

gfdA-A.niger          361 TCCAACCTACATGCTAACCCGTCGTTGGTTGACGCAGTCAAGGATAGTACCATCCTTGTC
gfdA-cDNA-A.niger     286 TCCAACCTACATGCTAACCCGTCGTTGGTTGACGCAGTCAAGGATAGTACCATCCTTGTC
gfdB-A.niger          327 GAAAACGTCGTAGCCGACCCAGATCTCAAGTCGGCCGTTAAAGATGCTACATTACTCGTC

gfdA-A.niger          421 TTCAACCTCCCCCACCAGTTTATCATCAAGACCTGTGAGCAGATCAAGGGCAAGATCCTG
gfdA-cDNA-A.niger     346 TTCAACCTCCCCCACCAGTTTATCATCAAGACCTGTGAGCAGATCAAGGGCAAGATCCTG
gfdB-A.niger          387 TTCAACCTCCCCCATCAGTTTATCGGCAAGACCCTCGATGGTGTTGTCGGGCACCATCTA

gfdA-A.niger          481 CCCTATGCGCGGGGTATCTCCTGCATCAAGGGTGTCGATGTGAACGAAGAAGGTATTCAT
gfdA-cDNA-A.niger     406 CCCTATGCGCGGGGTATCTCCTGCATCAAGGGTGTCGATGTGAACGAAGAAGGTATTCAT
gfdB-A.niger          447 CCCTATGCGCGAGGTATCTCTTGCATCAAGGGCGTCGATGTGTCCGACGGAACGGTGACC

gfdA-A.niger          541 CTCTTCTCCGAGACCATTGGCAAGATTCTGGGTATCTACTGCGGTGCTCTTTCTGGCGCG
gfdA-cDNA-A.niger     466 CTCTTCTCCGAGACCATTGGCAAGATTCTGGGTATCTACTGCGGTGCTCTTTCTGGCGCG
gfdB-A.niger          507 CTTCACTCGGAACTCATCATGGAGCGACTGAGCATCTACTGCGGTGCCTTGTCCGGCGCC

gfdA-A.niger          601 AACATTGCGAGCGAGGTAGCCCTGGAAAAATGGTCCGAGTCAAGCATTGCCTATGATCCT
gfdA-cDNA-A.niger     526 AACATTGCGAGCGAGGTAGCCCTGGAAAAATGGTCCGAGTCAAGCATTGCCTATGATCCT
gfdB-A.niger          567 AACATCGCCCCAGAGGTGGCCGCAGAGAAGTTTTGTGAGACGACCATTGGTTACGACACC

gfdA-A.niger          661 CCGCACATGGATTCCAAGGCGCCATCTCCCAATCGTTCCCCGTCTACGTCGACCGAGAAT
gfdA-cDNA-A.niger     586 CCGCACATGGATTCCAAGGCGCCATCTCCCAATCGTTCCCCGTCTACGTCGACCGAGAAT
gfdB-A.niger          627 CCTCCGATGGATGTGGAGGAGCG--TGATGGGTCGCCGAAGGAAAAC-TTAATCAAGATT

gfdA-A.niger          721 CTCGTTCAGTTCGAGCACAAGGATGTTTCCGGCCAACTATCACGAGTAAAGCTGCAAGCG
gfdA-cDNA-A.niger     646 CTCGTTCAGTTCGAGCACAAGGATGTTTCCGGCCAACTATCACGAGTAAAGCTGCAAGCG
gfdB-A.niger          684 GATGAGCAGCGGCAACATAAGACTA---------AACCCACGCATGTCAAATTACACCCG

gfdA-A.niger          781 CTGCCGTCCGACTACCCCCCTGTGGACCACGCCGTCCTGAAGTCGCTCTTCCATCGTCCG
gfdA-cDNA-A.niger     706 CTGCCGTCCGACTACCCCCCTGTGGACCACGCCGTCCTGAAGTCGCTCTTCCATCGTCCG
gfdB-A.niger          735 GTGCCAAAGGATCTCCCCGCTGTCGACGCTGAGCTGTGGGAGACTCTGTTTGCCCGCCCA

gfdA-A.niger          841 TATTTCCATATCCGTGTGGTGAGTGATGTCGCGGGTGTGTCGCTTGGAGGCGCCCTTAAG
gfdA-cDNA-A.niger     766 TATTTCCATATCCGTGTGGTGAGTGATGTCGCGGGTGTGTCGCTTGGAGGCGCCCTTAAG
gfdB-A.niger          795 TATTTCCATGTTAATCACGTTCGCGACGTTGCCGGGGTGGCCCTAGGGGGTGCACTGAAA

gfdA-A.niger          901 AACGTAGTTGCTCTTGCTGCCGGCTGGGTCGACGGCATGGGCTGGGGTGACAATGCCAAG
gfdA-cDNA-A.niger     826 AACGTAGTTGCTCTTGCTGCCGGCTGGGTCGACGGCATGGGCTGGGGTGACAATGCCAAG
gfdB-A.niger          855 AATATTGTAGCTTTAGCATCGGGGTTTGTCGCAGGAAAGGGCTGGGGAGAAAATGCCAAG

gfdA-A.niger          961 GCTGCCATCATGCGGGTTGGCCTTCTCGAGATGGTCAAGTTTGGTGAAAAGTTCTTCGGG
gfdA-cDNA-A.niger     886 GCTGCCATCATGCGGGTTGGCCTTCTCGAGATGGTCAAGTTTGGTGAAAAGTTCTTCGGG
gfdB-A.niger          915 GCTGCAATTATGCGTGTCGGCATCTTGGAGATGATCAAGTTTGGTCGGACGTGGTTTCCC
```

```
gfdA-A.niger       1021 GCCACCATCAATACTCGAACCTTCACTGAGGAAAGTGCGGGTGTTGCCGATCTGATTACT
gfdA-cDNA-A.niger   946 GCCACCATCAATACTCGAACCTTCACTGAGGAAAGTGCGGGTGTTGCCGATCTGATTACT
gfdB-A.niger        975 AAGTCTGTGGATGAGAGAACCTTTACCGAGGAGAGCGCGGGCCTAGCGGACGTGATCTCC

gfdA-A.niger       1081 AGCTGCAGCGGTGGCCGGAACTTTCGCTGCGCGAAACTCAGCATTGAG-AGGAAACAGTC
gfdA-cDNA-A.niger  1006 AGCTGCAGCGGTGGCCGGAACTTTCGCTGCGCGAAACTCAGCATTGAG-AGGAAACAGTC
gfdB-A.niger       1035 TCGTGTAGCGGTGGGCGGAACTTCCGGTCAGCTTGTCACGCAGTCGAGCAGGGGGTCAGC

gfdA-A.niger       1140 GATTGAGAAAGTCGAGGAGACGGAACTCAACGGCCAGAAGCTTCAGGGTACTTTGACTGC
gfdA-cDNA-A.niger  1065 GATTGAGAAAGTCGAGGAGACGGAACTCAACGGCCAGAAGCTTCAGGGTACTTTGACTGC
gfdB-A.niger       1095 G-TCAATGAAATCGAACAGAAGGAGCTCAACGGTCAGAAACTGCAGGGGACTTCGACGGC

gfdA-A.niger       1200 GGTTGAGGTAAACAACTTCCTGAAGAAGCAAGGCCTTGAGGAGGAATTTCCCTTGCTTAC
gfdA-cDNA-A.niger  1125 GGTTGAGGTAAACAACTTCCTGAAGAAGCAAGGCCTTGAGGAGGAATTTCCCTTGCTTAC
gfdB-A.niger       1154 CTACGCTGTTTATGACTTTCTCTCGAAGCACGACCAGTTGAAGGAATTTCCGCTGTTTGT

gfdA-A.niger       1260 GGCTGTGTACCGTGAGTAACCCAGTTCCTTTTCCCTTTATCTTGGTTTTTTGCCATCGAT
gfdA-cDNA-A.niger  1185 GGCTGTGTACCGT----------------------------------------------
gfdB-A.niger       1214 TGCTGTTCATGGTAAGTATTCTCTCCGGTTTTCTGTCCCTCACAATCTCTC---------

gfdA-A.niger       1320 GTACTGACCATAAAACAGGTGTTCTCGAGGGCAGTATGTCCGTTGGAGATATTCCTTCTT
gfdA-cDNA-A.niger  1199 ----------------------TTCTCGAGGGCAGTATGTCCGTTGGAGATATTCCTTCTT
gfdB-A.niger       1265 --ACTAACTTTCTGTCAGATATCCTCGAAGGGAAGTCTACTGTTGATGACCTCCCAGCAC

gfdA-A.niger       1380 ACATTGAGCGGTAA
gfdA-cDNA-A.niger  1238 ACATTGAGCGGTAA
gfdB-A.niger       1323 TGCTAG--------
```

Figure 6 - Alignment for maximal nucleotides similarities of *A. niger* gene *gfdA*, *gfdB* and the cDNA of *gfdA*. This alignment was produced using the clustalW program (http://align.genome.jp/clustalw/). Conserved residues have a black background.

From this alignment and confirming the prediction of HMM and Netgene2, we deduced that *gfdB* is a 1328 bp sequence starting at the position 1865740 of the scaffold 6 and having presumably two introns. The localisation of the introns, suggested in figure 7, was deduced from the alignment. Moreover, a TATA box and an element, CCGGGG, meeting the consensus sequence for the binding of CreA (SYGGRG) were localised upstream of *gfdB* respectively at the positions 1865843 and 1865863.

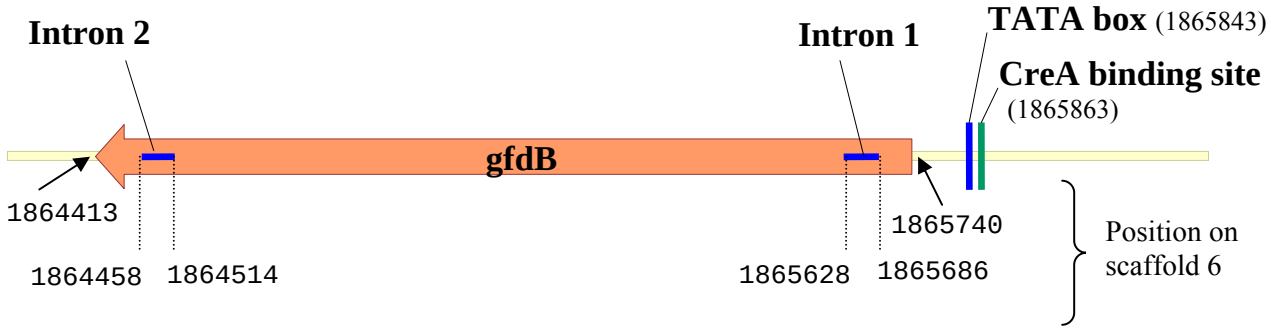


Figure 7 - Localisation and characterization of *gfdB* gene in *A. niger* genome sequence.

The 59% identity between the sequence of *gfdA* and *gfdB* and the similar organisation of these 2 genes indicates that these ORFs may have appeared due to gene duplication.

*Comparison of the deduced amino acid sequences of GfdA and GfdB with the amino acid sequences of G3PDH from other fungi.*

Alignment of the deduced amino acid sequences of the identified ORFs with the amino acid sequence of GfdA from *A. nidulans* (Fillinger et al., 2001), GfdA and GfdB from *A. fumigatus* (Nierman et al., 2005) and Gpd1 and Gpd2 from *S. cerevisiae* (Albertyn et al., 1994; Eriksson et al., 1995) is presented in Figure 8. A significant identity is observed throughout the sequences between the seven proteins, suggesting that the identified GfdA and GfdB encode for G3PDH.

```
GfdB-A.niger          1 ------------------------------------------------------------
GfdB-A.fumigatus      1 ------------------------------------------------------------
GfdA-A.niger          1 ------------------------------------------------------------
GfdA-A.nidulans       1 ------------------------------------------------------------
GfdA-A.fumigatus      1 ------------------------------------------------------------
GPD1-S.cerevisiae     1 ------------------------------------------------MSAAADRLNLT
GPD2-S.cerevisiae     1 MLAVRRLTRYTFLKRTHPVLYTRRAYKILPSRSTFLRRSLLQTQLHSKMTAHTNIKQHKH

GfdB-A.niger          1 ----------------MHEHTRKHKVAVVGSGNWGSTISKIVAENAREHSDLFEPEVRMW
GfdB-A.fumigatus      1 ------------MTSILNTAAADNLTHPVLNVTGKVISSIVTLKFYSQGHSGRLGKIGI
GfdA-A.niger          1 ------------MGSLGPYQRKHKITVVGSGNWGCAIAKIVAENAASNPAIFEEKVEMW
GfdA-A.nidulans       1 ------------MGSLGPYKQKHKVTVVGSGNWGTAIAKIVAENTASNPAVFEKDVQMW
GfdA-A.fumigatus      1 ------------MGSLSPHLRKHKVAVVGSGNWGTAIAKIVAENTASNDSLFERDVEMW
GPD1-S.cerevisiae    12 SGHLNAGRKRSSSSVSLKAAEKPFKVTVIGSGNWGTTIAKVVAENCKGYPEVFAPIVQMW
GPD2-S.cerevisiae    61 CHEDHPIRRSDSAVSIVHLKRAPFKVTVIGSGNWGTTIAKVIAENTELHSHIFEPEVRMW

GfdB-A.niger         45 VFEEEIEIPESSKHHSKLGGQK------HKLTEVINGVHENVKYLPDIALPENVVADPDL
GfdB-A.fumigatus     48 GIDDRQNPGRKHSRTSRCLRRRGADVGTHKLTDIINRVHENVKYLPGTALPSNVVANPDL
GfdA-A.niger         48 VFEEKVEISKDSRHYDPSSPLCQ---GPQNLTDVINQKHENIKYLPGITLPSNLHANPSL
GfdA-A.nidulans      48 VFEEKVEIPKSSKHYDPASSLCQ---GPQNLTDIINHTHENIKYLPGITLPENLIANPSL
GfdA-A.fumigatus     48 VFEEKVEIPKNSRHYDPSSPLCQ---GPQNLTEIINKTHENVKYLPGIALPENIHANPSV
GPD1-S.cerevisiae    72 VFEEEIN-------------------GEKLTEIINTRHQNVKYLPGITLPDNLVANPDL
GPD2-S.cerevisiae   121 VFDEKIG-------------------DENLTDIINTRHQNVKYLPNIDLPHNLVADPDL

GfdB-A.niger         99 KSAVKDATLLVFNLPHQFIGKTLDGVVGHHLPYARGISCIKGVDVSDGTVTLHSELIMER
GfdB-A.fumigatus    108 RAAVKDATILVFNLPHQFIDKTLEQMKGHHLPYARAVSCVKGVEVANGRVTLFSELIMEQ
GfdA-A.niger        105 VDAVKDSTILVFNLPHQFIIKTCEQIKGKILPYARGISCIKGVDVNEEGIHLFSETIGKI
GfdA-A.nidulans     105 VDAVQDSTILVFNLPHQFIINICEQIKGKIVPYARGISCIKGVDVNEEGVHLFSETIGKI
GfdA-A.fumigatus    105 VDAVKDSTILIFNLPHQFINKTCEQIKGKILPYARGISCIKGVDVTEEGVSLFSETIGKT
GPD1-S.cerevisiae   112 IDSVKDVDIIVFNIPHQFLPRICSQLKGHVDSHVRAISCLKGFEVGAKGVQLLSSYITEE
GPD2-S.cerevisiae   161 LHSIKGADILVFNIPHQFLPNIVKQLQGHVAPHVRAISCLKGFELGSKGVQLLSSYVTDE

GfdB-A.niger        159 LSIYCGALSGANIAPEVAAEKFCETTIGYDTPPMDVEERDG-----SPKENLIKIDEQRQ
GfdB-A.fumigatus    168 LGIYCGSLSGANIAPEVAAEEFCETTIGYSPPPMDLDAKDE-----LSPD----------
GfdA-A.niger        165 LGIYCGALSGANIASEVALEKWSESSIAYDPPHMDSKAPSP-NRSPSTSTENLVQFEHKD
```

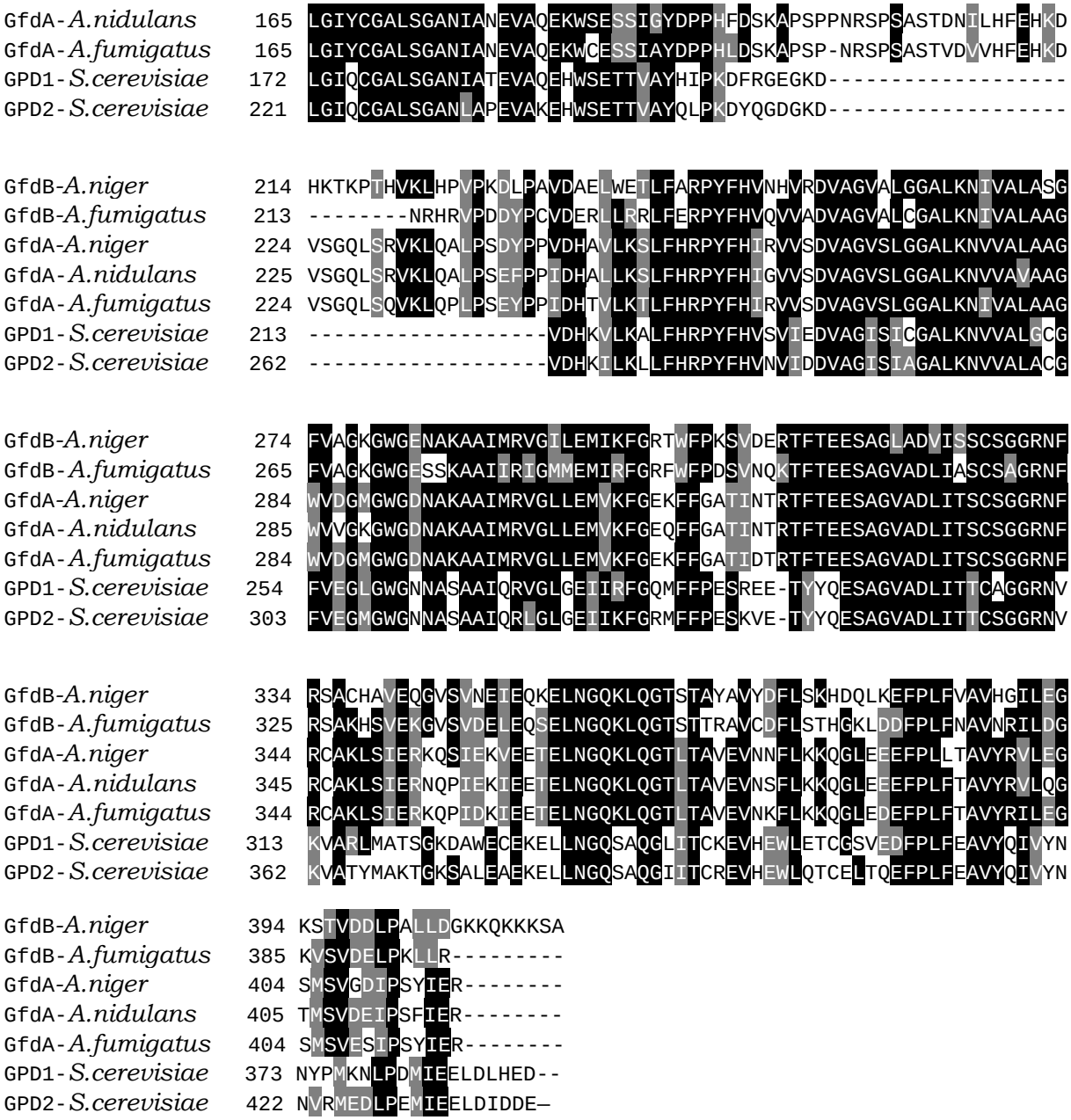


Figure 8 - Alignment for maximum amino acid similarities of the *A. niger* GfdA and GfdB protein with the amino acid sequence of G3PDH from: *A. nidulans* (GfdA-A.nidulans, (Fillinger et al., 2001)), A. fumigatus (GfdA-A.fumigatus and GfdB-A.fumigatus, (Nierman et al., 2005)) and S. cerevisiae (GPD1, (Albertyn et al., 1994), GPD2, (Eriksson et al., 1995)). This alignment was produced using the clustalW program (http://align.genome.jp/clustalw/). Conserved residues have a black background.

*Study of the expression of gfdA and gfdB*

Expression of the two *gfd* genes was studied in order to check whether both ORFs were expressed in the wild type strain, *A. niger BO1,* during the batch cultivation conditions described in material and methods. The expression of *gfdA* and *gfdB* was analysed in the exponential growth phase and in the oxygen limitation phase by Reverse Transcription PCR (RT-PCR) (Figure 9). Concerning *gfdA*, a band at 668 bp was present at both

conditions, indicating the expression of this gene both in the exponential phase and in the oxygen limitation phase. For *gfdB*, the absence of a band at 600 bp indicated that *gfdB* was expressed neither in the exponential phase nor in the oxygen limitation phase. The band at 900 bp observed in line 3, figure 9, was purified and sequenced. The sequence showed no homology with *gfdB*, confirming the fact that *gfdB* was not expressed under these cultivation conditions. To look further into the physiological function of G3PDH in *A. niger* only *gfdA* was deleted, and the influence of such a deletion on the expression of *gfdB* is discussed later.

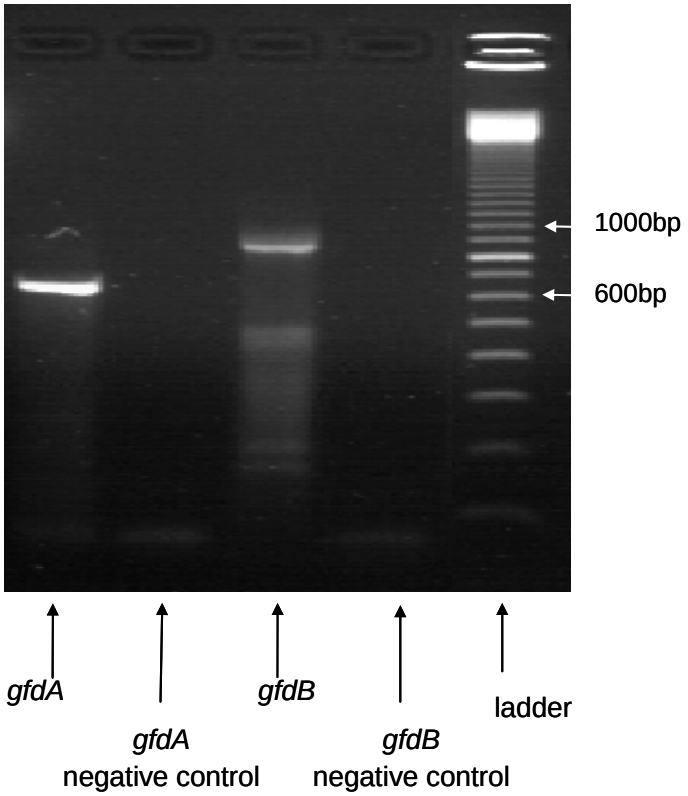


Figure 9 - Results of RT-PCR performed on *A. niger BO1* mRNA from exponential growth. The negative control has been run on the RNA samples omitting the reverse transcriptase step to check for any contamination with DNA. For more information see chapter material and methods.

### b. Deletion of *mpdA* and *gfdA*

#### *Investigation of the genic organisation in the areas to be deleted*

The deletion of *mpdA* and *gfdA* could affect the expression of any other genes, which could be located, for example, on the complementary DNA strand. Two genomic fragments containing the *mpdA* and *gfdA* genes were therefore submitted for a homology search using the NCBI database. This analysis did not localise any known gene except *mpdA* and *gfdA* in the areas to be deleted, which suggested that the deletions of *mpdA* and *gfdA* should not affect the transcription of other genes.

***Deletion strategy***

To delete *mpdA*, *A. niger Jroy3* was transformed with pAudd10. Out of 11 transformants checked by PCR, 4 had the right pattern. Gene replacement was confirmed by Southern analysis (Appendix III): one strain was picked up and named A10. This construction led to the deletion of 102 nucleotides from the promoter area and 658 nucleotides from *mpdA* gene, leaving the last nucleotides of the *mpdA* gene intact in the genome (Figure 3). This deletion strategy was based on the fact that the sequence available at the time of the deletion process was based only on a 4x coverage and therefore only 417 nucleotides were available after the gene.

To delete *gfdA*, *A. niger Jroy3* was transformed with pAudd7. Out of 7 transformants checked by PCR, 3 were found to have the right pattern. Gene replacement was confirmed by Southern analysis for the 3 strains, and one strain was selected and named A7 (Appendix IV). This construction led to a deletion of the last 861 nucleotides, leaving the promotor and the 534 first nucleotides in the genome. The reason for this strategy was that, at the time of the *gfdA* deletion process, the sequence available had only a 4x coverage and no more than 520 nucleotides were available upstream of the gene. A total deletion using only 511 bp homology upstream the *gfdA* gene did not lead to any correct mutant within the 40 mutants checked, although the downstream area was more than 2 kb long. However, the analysis of the protein domain using the server Pfam (http://www.sanger.ac.uk/Software/Pfam/) shows that the part deleted contains the substrate binding domain, meaning that the truncated protein should not be functional.

To delete both the *mpdA* and *gfdA* genes, *A. niger Jroy3* was first transformed with pAudd8. Three strains checked by PCR were found to have the correct integration. Gene replacement was confirmed by Southern analysis: one strain was selected and named A8 (Appendix III). Excision of the *pyrG* marker through recombination between homologous flanking regions was achieved by selection for growth in the presence of 5-fluoroorotic acid (5-FOA) (Nielsen et al., 2006). Two 5-FOA resistants were analyzed by Southern analysis and showed the expected pattern (Appendix V). One of the transformants named $A8^{-}$ was used for transformation using the linearised vector pAudd7. Out of 7 transformants checked by PCR, 2 were found to have the right pattern. Gene replacement was confirmed by Southern analysis, and one strain was selected and named A87 (Appendix IV and V).

### c. Physiology and morphology of the mutants in batch cultivations

To check the influence of *pyrG* insertion on the growth of the mutants, shake flasks were run with and without uridine. The ratios of the biomass concentration of the wild type*, A. niger BO1*, versus the biomass concentration of the mutants were the same with and without uridine, showing that there was no effect of *pyrG* insertion on the biomass germination and growth (data not shown).

#### *mpdA mutant*

In the batch cultures, the lag phase of the *mpdA* mutant was delayed for 7 hours (corresponding to a 60% increase). During the exponential phase, the maximum specific growth rate decreased by 39% (Table 3).

Table 3 - Specific rates in the exponential growth phase (μmol/g DW/h). Average and standard deviation were calculated on 4 sets of samples, except concerning malic acid.

| | | wt | | mpdA | | mpdA-gfdA | | gfdA | |
|---|---|---|---|---|---|---|---|---|---|
| | | AVE | STD | AVE | STD | AVE | STD | AVE | STD |
| Biomass (h-1) | | **0.27** | 0.02 | **0.16** | 0.00 | **0.13** | 0.00 | **0.16** | 0.01 |
| polyols | Glycerol | **175.93** | 8.84 | **313.39** | 41.32 | **438.90** | 48.86 | **385.07** | 22.35 |
| | Erythriol | **3.67** | 2.40 | **0.96** | 0.91 | **28.63** | 1.36 | **27.96** | 5.49 |
| | Mannitol | **0.00** | 0.00 | **0.00** | 0.00 | **0.00** | 0.00 | **0.00** | 0.00 |
| acids | Succinic | **0.00** | 0.00 | **114.48** | 33.62 | **33.67** | 7.43 | **43.76** | 11.87 |
| | Fumaric | **0.00** | 0.00 | **0.85** | 0.00 | **0.85** | 0.07 | **0.00** | 0.00 |
| | Citric | **22.59** | 1.32 | **107.00** | 13.38 | **48.57** | 7.41 | **70.13** | 24.27 |
| | Pyruvic | **0.23** | 0.32 | **60.29** | 12.94 | **12.40** | 0.31 | **2.52** | 2.85 |
| | Oxalic | **0.00** | 0.00 | **1.84** | 0.13 | **18.90** | 3.84 | **0.00** | 0.00 |
| | Malic | **3.42** | n.d. | **642.58** | n.d. | **13.96** | n.d. | **9.79** | 3.66 |
| $O_2$ | | **-6117.64** | 298.66 | **-5922.54** | 245.95 | **-4971.95** | 71.96 | **-4920.90** | 46.06 |
| $CO_2$ | | **6103.62** | 534.53 | **5754.67** | 60.35 | **5230.59** | 150.15 | **5292.65** | 16.27 |
| glucose | | **-3492.80** | 204.30 | **-2878.89** | 243.79 | **-2219.75** | 56.14 | **-2430.05** | 7.47 |
| $YO_2$ | | **0.29** | 0.00 | **0.34** | 0.01 | **0.37** | 0.01 | **0.34** | 0.00 |
| $YCO_2$ | | **0.29** | 0.01 | **0.33** | 0.03 | **0.39** | 0.02 | **0.36** | 0.00 |
| RQ | | **1.00** | 0.04 | **0.97** | 0.05 | **1.05** | 0.01 | **1.08** | 0.01 |

In the exponential growth phase the morphology of the mutant was quite similar to that of the wild type strain. The diameters of the hyphae were about 3.7 μm in the subapical compartment for both strains. However, the hyphae of the mutant were found to swell in the branching regions (Table 4). The presence of ballon-like structures in the hyphae was observed for the wild type and the mutant, as well as the presence of oval vacuoles. This is probably linked to the high glucose concentration causing osmotic pressure on the hyphae. However, the mutant had bigger vacuoles implying a lower percentage of cytoplasm, which represents the active part of the hyphae (Appendix VI).

Table 4 - Diameter (μm) of the wild type and mutant strains during batch cultivations.

| | exponential phase | | $O_2$ limitation phase | |
|---|---|---|---|---|
| | AVE | SD | AVE | SD |
| wt | **3.65** | 0.35 | **3.32** | 0.37 |
| mpdA | **3.75** | 0.48 | **2.36** | 0.55 |
| gfdA | **3.80** | 0.53 | **3.20** | 0.39 |
| mpdA-gfdA | **3.91** | 0.70 | **2.80** | 0.54 |

During oxygen limitation, the diameter of both strains decreased significantly. The diameter of the wild type decreased to 3.3 μm, while the diameter of the *mpdA* mutant decreased to 2.4 μm. The hyphae of the mutant were thinner and filled with long vacuoles. Fragmentation of the hyphae was observed in the oxygen limitation phase for both strains but it was more pronounced for the *mpdA* mutant (Appendix VI). At this time, the specific growth rate was 60% lower for the *mpdA* strain (Table 5).

Table 5 - Specific rates in the oxygen limitation phase (μmol/g DW/h). Average and standard deviation were calculated based on 4 sets of samples, except for the wild type (2 sets) and malic acid (1-2 sets).

| | | wt | | mpdA | | mpdA-gfdA | | gfdA | |
|---|---|---|---|---|---|---|---|---|---|
| | | AVE | STD | AVE | STD | AVE | STD | AVE | STD |
| Biomass (h-1) | | **0.02** | 0.00 | **0.01** | 0.00 | **0.01** | 0.00 | **0.02** | 0.00 |
| polyols | Glycerol | **152.85** | 3.05 | **115.84** | 1.33 | **95.42** | 1.28 | **111.91** | 15.08 |
| | Erythriol | **31.38** | 0.55 | **45.71** | 4.32 | **34.88** | 0.78 | **18.64** | 0.09 |
| | Mannitol | **82.24** | 0.52 | **4.03** | 0.53 | **2.96** | 0.73 | **58.03** | 0.81 |
| acids | Succinic | **1.24** | 0.20 | **-2.10** | 0.45 | **3.68** | 1.73 | **9.01** | 0.35 |
| | Fumaric | **0.58** | 0.01 | **2.03** | 0.28 | **1.63** | 0.16 | **1.39** | 0.63 |
| | Citric | **0.29** | 0.01 | **-2.35** | 0.65 | **0.46** | 0.21 | **2.42** | 0.05 |
| | Pyruvic | **1.20** | 0.17 | **-3.31** | 0.18 | **-0.13** | 0.05 | **1.08** | 0.12 |
| | Oxalic | **0.07** | 0.01 | **0.64** | 0.06 | **0.12** | 0.11 | **-0.03** | 0.35 |
| | Malic | **1.62** | n.d. | **13.66** | n.d. | **7.25** | n.d. | **8.58** | 0.66 |
| $O_2$ | | **-809.74** | 7.95 | **-801.73** | 12.81 | **-874.75** | 85.67 | **-1030.87** | 15.48 |
| $CO_2$ | | **1047.83** | 15.86 | **917.08** | 7.74 | **1000.17** | 100.12 | **1351.60** | 2.38 |
| glucose | | **-473.78** | 7.93 | **-362.30** | 38.21 | **-364.35** | 35.11 | **-504.70** | 29.93 |
| $YO_2$ | | **0.28** | 0.01 | **0.37** | 0.05 | **0.40** | 0.09 | **0.34** | 0.02 |
| $YCO_2$ | | **0.37** | 0.00 | **0.42** | 0.05 | **0.46** | 0.10 | **0.45** | 0.03 |
| RQ | | **1.29** | 0.03 | **1.14** | 0.01 | **1.14** | 0.00 | **1.31** | 0.02 |

All along the fermentations, the lower specific growth rate led to a lower specific consumption rate of glucose and oxygen, as well as a lower specific production rate of carbon dioxide. However, the yields of oxygen and carbon dioxide increased both in the exponential phase and the oxygen limitation phase. The respiratory quotient (RQ) for both strains was close to 1 in the exponential phase and increased in the oxygen limitation phase to 1.25 for the wild type and 1.14 for the *mpdA* deleted strain.

Concerning metabolite production, the deletion of *mpdA* did not suppress the production of mannitol (Table 5). To check the provenance of this polyol, the MPD activity was

measured in the wild type strain and in the *mpdA* mutant. No MPD activity was found in the mutant confirming the deletion of *mpdA* and suggesting a production of mannitol via (an)other pathway(s). In the wild type strain, an activity of 40 mU/mg protein (±2) was measured in the exponential growth phase, while in the oxygen limitation phase the activity increased to 280 mU/mg protein (±6), underlying the importance of this enzyme during oxygen limitation. The deletion of *mpdA* increased the production of glycerol in the exponential growth phase, while there was a decrease in its production during the oxygen limitation phase. Erythritol is mainly produced in the oxygen limitation phase and its production increased by 46% in the *mpdA* mutant. Concerning the organic acid production, the specific production rate of all organic acids dramatically increased in the exponential growth phase, especially concerning the specific production rate of succinic, pyruvic and malic acids. From the exponential to the oxygen limitation phase, the production of organic acids in the wild type strain generally increased, while there was a dramatic carbon flux redistribution in the *mpdA* mutant. Indeed succinic, citric and pyruvic acid were reconsumed, while the production of fumaric acid was increased. Oxalic and malic acid production decreased during the shift from exponential to oxygen-limited growth, but their specific production rates were 8 times higher in the mutant than in the wild type strain.

***gfdA mutant***

The lag phase was delayed for 5 hours in the mutant (corresponding to a 40% increase), and the maximum specific growth rate was 39% lower. In the exponential growth phase, the diameter of the hyphae in the subapical compartment did not change significantly although the *gfdA* mutant had irregular diameters with swollen hyphae at the vicinity of branching area (Table 4). As mentioned for the wild type and the *mpdA* strains, ballon-like structures were also observed in the *gfdA* mutant. Similarly to the *mpdA* strain, an increased content of vacuoles was also observed in the *gfdA* strain compared to the wild type (Appendix VI). During the oxygen limitation phase the diameter of the hyphae decreased from 3.80 to 3.19 μm, which was similar to the diameter of the wild type (Table 4). A lot of small vacuoles were present in the mycelia associated with the presence of many fragmented hyphae (Appendix VI). However, in the period of oxygen limitation, the specific growth rate was similar in both strains.

The specific consumption rate of oxygen and the specific production rate of carbon dioxide were lower in the exponential phase and higher during oxygen limitation in the *gfdA* strain compared to the wild type. However, the carbon dioxide and oxygen yields increased both in the exponential phase and in the oxygen limitation phase, showing that there was a redistribution of the carbon flux and an increase in the mitochondrial activ-

ity per glucose consumed in the mutant. The specific consumption rate of glucose of the mutant was lower in the exponential phase and similar to the wild type rate in the oxygen limitation phase. Moreover no important variations in the RQ were observed between the two strains.

Concerning the metabolites, a deletion of *gfdA* led, in the exponential growth phase, to an increase by a factor 2 of the polyol specific production rate, including the rate of glycerol production, while in the oxygen limitation phase, the specific production rate of all polyols decreased. The production of organic acids along the fermentations was generally higher in the *gfdA* mutant especially concerning succinic, citric and malic acids.

***mpdA and gfdA double mutant***

The lag phase was delayed for 7 hours in the mutant (corresponding to a 60% increase) and the maximum specific growth rate decreased by 50%. The deletion of both genes had a substantial effect on the morphology of the hyphae. The diameter of the hyphae was very irregular with several swollen areas, in particularly near the branching region. The average value of the diameter in the subapical compartment was, however, not significantly different from that of the wild type. In the mutant the vacuoles occupied the majority of the hyphae volume (Appendix VI). During oxygen limitation the diameter of the hyphae decreased significantly from 3.91 to 2.8 μm. As for the *mpdA* deletion strain, the hyphae diameter decreased at the tips. A large number of small and long vacuoles were also present during oxygen limitation. Hyphal fragmentation started at the beginning of the oxygen limitation phase (seen as fragmented hyphae in the microscope) and after 35 h of oxygen limitation most of the hyphae were fragmented (Appendix VI). During this period, the specific growth rate was 37% lower than for the wild type. For the double deletion mutant, the specific consumption rate of glucose was 30-40% lower in both the exponential and the oxygen limitation phase. The specific consumption rates of glucose and oxygen, as well as the specific production rate of carbon dioxide and polyols, were similar to what was observed for the *gfdA* mutant in the exponential phase, and the RQ was about 1.05. However, in the oxygen limitation phase, the mutant was more similar to the *mpdA* mutant, in particular with respect to polyol production and RQ. The specific production rates of organic acids were generally higher than for the wild type during both phases, and for most of the organic acids, production levels were between what was observed for the *mpdA* and *gfdA* strains.

### d. G3PDH transcript and enzyme activity

The expression of *gfdA* and *gfdB* were analyzed in the *mpdA*, *gfdA* and *mpdA_gfdA* mutants by RT-PCR in both the exponential growth phase and the oxygen limitation phase.

For each strain, the results were identical in the two growth phases. The RT-PCR results of *gfdA* and *mpdA-gfdA* mutants were also similar.

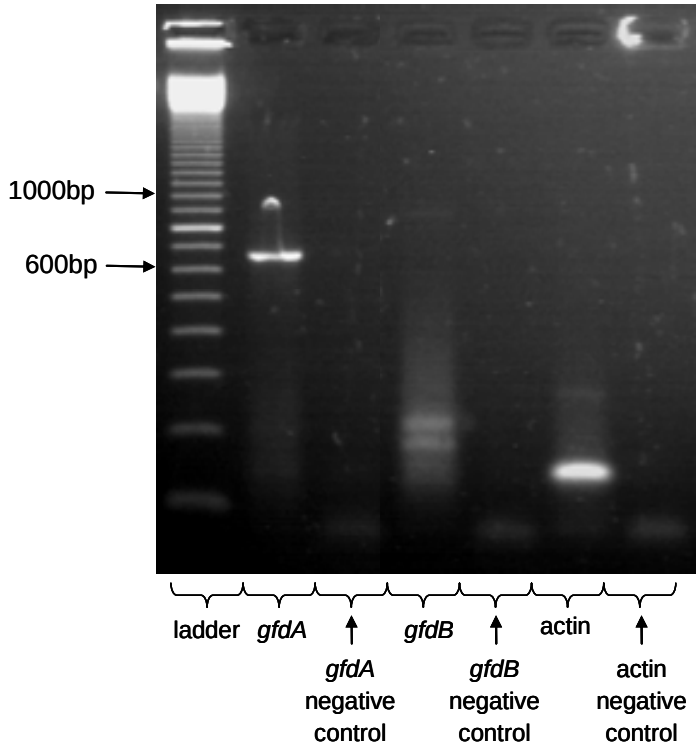


Figure 10 - Results of RT-PCR performed on *A. niger A10* mRNA from the exponential growth phase. The negative control was run on the RNA samples omitting the reverse transcriptase step to check for any contamination with DNA. The lines 6 and 7, represent actin, which was used as positive control. For more information see material and methods.

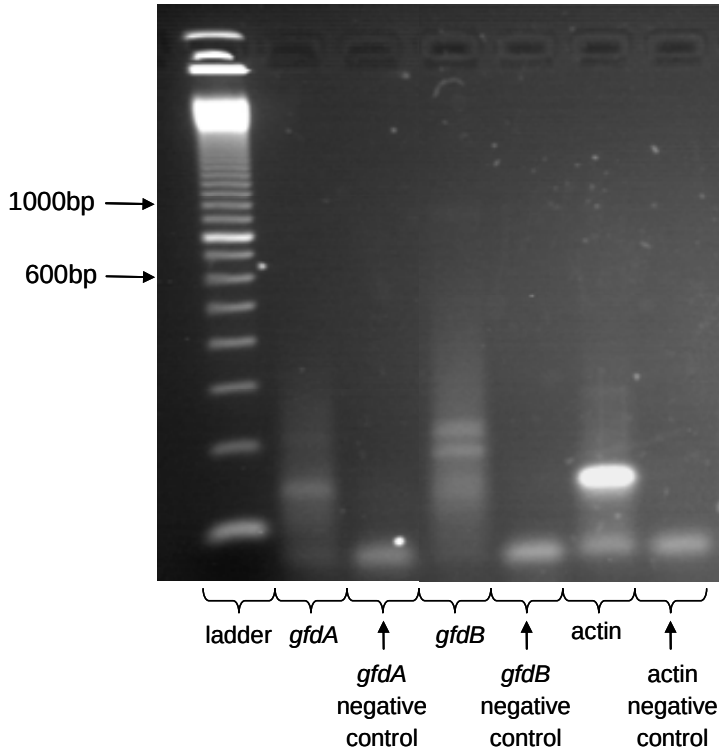


Figure 11 - Results of RT-PCR performed on *A. niger A7* mRNA from the exponential growth phase. The negative control was run on the RNA samples omitting the reverse transcriptase step to check for any contamination with DNA. The lines 6 and 7, represent actin, which was used as positive control. For more information see material and methods.

The transcription of *gfdB* would give a band at 650 bp in the lines 4 of figures 10 and 11. The absence of a PCR product leads to the conclusion that *gfdB* is not expressed in any of the strains at the cultivation conditions tested. In the second round of RT-PCR the expression of *gfdA* was observed in the *mpdA* mutant but not in the *gfdA* and *mpdA-gfdA* mutants as the DNA fragment hybridising with the primer RT4-gfdA has been removed in these two strains. However, in the first round of PCR the use of a sens primer located at the beginning of gfdA gene together with a poly-dT primer gave a 850 bp PCR product, sharing homology with gfdA sequence (data not shown). This indicates the presence of *gfdA* mRNA, which is likely to be transcribed from the truncated gene. To check whether this truncated transcript could result in enzyme activity, G3PDH activity was measured in the wild type strain and in the *gfdA* and *mpdA-gfdA* mutants. No activity could be detected in neither the *gfdA* nor the *mpdA-gfdA* mutants, confirming the results of the previous Pfam analysis mentioned p.74, which predicted the absence of the substrate binding domain in the truncated protein. In the wild type strain, an activity of 36 mU/mg protein (±1) and 22 mU/mg protein (±2) was measured in the exponential and in the oxygen limitation phases, respectively.

### e. Complementary study on the role of *mpdA* and *gfdA* in *A. niger BO1*.

Table 6 shows the influence of the carbon source and osmotic pressure on the diameter of the colonies of the wild type and the mutant strains. The purpose of this study was to gain information on the metabolism of the different mutants. The collected data indicate that the growth of all the mutants on plates was affected by an increase in the osmotic pressure when glucose was the carbon source. However, differences were observed for growth on glycerol, and the *mpdA* mutant had enhanced growth on mannitol. Moreover, growth of the *mpdA* mutant on sorbitol was quite similar to the growth on glucose, although sorbitol increases the osmotic pressure of the medium and hereby decreases the growth of the wild type. The growth of the *mpdA* mutant was also enhanced on a mixture of fructose and lactose. These observations indicate the importance of mannitol, sorbitol and fructose in the *A. niger* metabolism. Indeed, mannitol and sorbitol can both be converted into fructose via the MTD and the sorbitol dehydrogenase, and fructose can be further converted to mannose (via the mannose isomerase), which is an important compound for cell wall biosynthesis, (Johnston, 1965). Furthermore, the concentration of mannitol in the spores was important for the germination process, as the spores harvested from mannitol plates were growing faster.

Table 6 - Colony diameters (in cm) of the wild type and mutants on different carbon sources. (b) and (c) indicate that the spores for inoculation were harvested from minimal medium plates using glycerol or mannitol as carbon source, respectively.

| C source | Supplementation | wt | A10 | A87 | A7 |
|---|---|---|---|---|---|
| Glucose | no | 2.4 | 0.9 | 0.5 | 0.7 |
| | 1M NaCl | 2.2 | 0.5 | 0.4 | 0.4 |
| Glycerol | no | 2.0 | 0.2 | 0.2 | 0.4 |
| | 1M NaCl | 1.8 | 0.3 | 0.3 | 0.4 |
| Glycerol (b) | no | n.d. | 0.2 | 0.2 | 0.5 |
| | 1M NaCl | n.d. | 0.4 | 0.3 | 0.4 |
| 2% Lactose | no | 1.1 | 0.8 | 0.3 | 0.4 |
| | 10mM Glycerol | 1.6 | 0.7 | 0.6 | 1.1 |
| | 10mM Fructose | 1.4 | 1.2 | 0.2 | 0.3 |
| Mannitol | no | 2.4 | 1.2 | 0.2 | 0.3 |
| Mannitol (c) | no | 2.3 | 1.7 | 0.4 | 0.4 |
| Fructose | no | 1.7 | 0.9 | 0.3 | 0.6 |
| Sorbitol | no | 1.7 | 0.8 | 0.3 | 0.3 |

The growth of the *gfdA* mutant was enhanced by the addition of glycerol on lactose indicating the importance of the glycerol pathway on *A. niger* metabolism.

The double mutation highly affected the growth on all carbon sources tested, underlining the importance of *mpdA* and *gfdA* genes in the metabolism of *A. niger BO1*.

The sporulation of all the mutants was retarded compared to the wild type, but the addition of 1 M NaCl accelerated the sporulation process.

## 4. Discussion

### a. Role of the *mpdA* gene

Deletion of *mpdA* had broad effects on the metabolism of *A. niger*. The different growth characteristics on plates, the longer lag phase during the fermentations and the decrease of the *mpdA* mutant specific growth rate underline the importance of MPD during the complete life cycle of *A. niger*. The MPD enzyme is involved in the production of mannitol, which is required for the protection of spores against high temperature and oxidative conditions (Ruijter et al., 2003). Also during germination this enzyme is important due to its role in providing storage compounds and balancing the redox metabolism (Blumenthal, 1976; Witteveen and Visser, 1995). Interestingly, a previous study has shown that a deletion of *mpdA* in *A. niger N400* does not delay germination, growth nor sporulation on minimal medium containing 1% glucose and does not affect the growth of the mutant on minimal medium plates containing 1% carbon source such as glucose, fructose, mannitol, glycerol, and acetate (Ruijter et al., 2003). The growth characteristics of the mutant *A. niger A10*, constructed here, were found to be quite different from the growth characteristics of the *mpdA* mutant described by Ruijter et al. (2003). The reason could be the use of two different *A. niger* wild types showing differences in the synthesis of fructose. Indeed, in the strains derived from *A. niger BO1,* fructose seems to have an important function and seems to be mainly produced by the mannitol catabolic pathway. This hypothesis is mainly based on the growth characteristics of the mutants on plates, as the growth of the *mpdA* mutant is enhanced by the presence of fructose and mannitol. Moreover, the ratio "colony diameter of *mpdA* mutant versus colony diameter of wild type" is increased on sorbitol, metabolite which can be converted into fructose via the sorbitol dehydrogenase. This hypothesis is also supported by the batch cultivations results, where the maximum specific growth rate of *A. niger A10* is highly affected even though, in this cultivation phase, no mannitol could be detected in the wild type culture. This underlines the importance of MPD itself and/or the importance of one of the components of the mannitol pathway during the exponential growth phase. This compound is likely to be fructose, which could then be further directed towards mannose production and cell wall synthesis (Johnston, 1965). The morphology of the mutant also supports this hypothesis as the presence of swollen hyphae in the vicinity of the branching area could be explained by an altered cell wall composition. Another hypothesis could be that the mannitol pathway is used as a route to produce NADPH at the expense of NADH and ATP. The presence of such a cycle in

fungi has been previously proposed by Hult and Gatenbeck (1978), but rejected by Ruijter et al. (2003) and Singh et al. (1988).

The increase of the carbon dioxide yield in the exponential phase is a sign of a redistribution of the carbon flux in favour of the PP pathway and/or the TCA cycle. The increase of oxygen consumption per unit of metabolised glucose indicates that there is an increase in the mitochondrial activity in the *mpdA* mutant. This may be linked to an increase in the energetic need for biomass production and maintenance and/or the loss of NADH oxidation by MPD. A role of MPD in NADH oxidation, even when the oxygen supply is sufficient, is suggested by the substantial increase in organic acid production in the mutant. Indeed the accumulation of pyruvic, citric, succinic, fumaric, malic and oxalic acids indicates a deregulation of the TCA cycle, which may be linked to an intracellular accumulation of NADH. The study of Singh et al. (1988) has suggested a role of MPD as a transporter of reducing equivalents from the cytosol to the mitochondria. More precisely, these authors have localised the MPD both in the cytosol and in the mitochondrial matrix, showing the existence of two isoenzymes with different structures in *A. nidulans*. This is in contradiction with our study and the study of Ruijter et al. (2003), as no MPD activity was detected after the deletion of *mpdA*. Moreover, the annotation of the *A. nidulans* genome resulted only in one ORF encoding for MPD. The difference between these studies is difficult to explain but the present work clearly indicates that MPD has an influence on mitochondrial reactions. Malic acid, the most abundant organic acid produced by the *mpdA* mutant can be synthesised both by the TCA cycle in the mitochondria and in the cytosol. Its production from pyruvic acid in the cytosol via OAA involves the reoxidation of NADH and its production and excretion could be due to a cytosolic NADH accumulation when MPD is inactive. It could also represent a redox shuttle between the cytosol and the mitochondria. However, these different hypotheses do not explain why the double deletion mutant *mpdA-gfdA* produced lower amounts of organic acids than the *mpdA* mutant in the exponential phase.

During oxygen limitation, the accumulation of NADH in the wild type strain leads to a reduction in the specific growth rate and to an accumulation of mannitol, the production of which involves NADH reoxidation (Diano et al., 2006). The carbon flux distribution changes, which leads to an increase in the carbon dioxide yield, while the oxygen yield stays constant. The RQ therefore increased to 1.25. A lower increase in the RQ was observed for the single deletion mutant A10 and the double deletion mutant A87, and this is due to a high oxygen yield in these strains. Thus, in these mutants more oxygen is consumed per unit of metabolised glucose, indicating an increase of NADH oxidation by the respiratory chain. This may be due to the deletion of MPD, which represents an alternative NADH sink (Diano et al., submitted). Moreover, this deletion has drastic effects on the cell metabolism, i.e. the specific growth rate and the specific

consumption rate of glucose decrease, while the production of metabolites changes. Low mannitol amounts are still detected. The production of mannitol in an *mpdA* deleted strain has also been observed in conidiospores of a derived strain of *A. niger N400* (Ruijter et al., 2003). In such conditions, mannitol may come from another yet non identified pathway or, more probably, in this case, from the activity of non specific alcohol dehydrogenase(s), as the mannitol specific production rate of the *mpdA* mutant represents less than 5% of the wild type specific production rate.

Finally, the stress caused by the deletion of *mpdA* during oxygen limitation is also reflected on the morphology. Indeed, a lack of oxygen leads to a decrease in the diameter of the wild type hyphae and to an increase in the fraction of vacuoles, causing more hyphal fragmentation (Wongwicharn et al., 1999). A deletion of *mpdA* exaggerated the effect of a lack of oxygen with a further decrease in the hyphal diameter and increased vacuole formation, as well as a high level of hyphal fragmentation. However, it should be noticed that the decrease in the hyphal diameter is not directly linked to the limitation of oxygen, but seems to be related to a diminution of the specific growth rate (Diano et al., submitted).

### b. Role of the *gfdA* gene

A deletion of *gfdA* increases the lag phase and decreases the maximum specific growth rate. The glycerol pathway is known to have an important role during germination in filamentous fungi. During the first hours of germination, glycerol accumulates, while the concentration of others polyols decreases (d'Enfert and Fontaine, 1997; Witteveen and Visser, 1995). Glycerol probably serves in increasing the intracellular turgor potential necessary for germination and hyphal growth. However a deletion of *gfdA* in *A. nidulans* does not affect the intracellular concentration of glycerol during germination, although it prolongs the lag phase by 40% (Fillinger et al., 2001). The delay in the lag phase is thereby due to another factor than a deficiency in glycerol accumulation. Moreover, during vegetative growth on minimal media, Fillinger et al. (2001) noticed a diminution of intracellular glycerol concentration both at normal and high osmotic pressures in the *gfdA* mutant. A few years later, De Vries et al. (2003) showed that the difference observed in intracellular glycerol concentration at high osmotic pressures is not due directly to the deletion of *gfdA*, as the main pathway active under these conditions is the one via DHA (Figure 2). The low level of intracellular glycerol observed by fillinger et al. in the *gfdA* mutant is therefore likely to be caused by secondary effects. Indeed the *gfdA* mutant has a low level of G3P, which has been found to affect the cell wall synthesis (Fillinger et al., 2001). A deletion of *gfdA* leads to the formation of swallowed hyphae, particularly in the branching region, which is often seen during growth on media with high osmotic pressures (Fillinger et al., 2001). The deletion of *gfdA* could

therefore result in the presence of a high osmotic pressure inside the cell, which could lead to an increase in glycerol excretion and this could explain the diminution of intracellular glycerol observed by Fillinger et al. (2001) as well as the increase of the apparent specific production rate of glycerol and erythritol observed in this study. Indeed, both these compounds play a role in osmoregulation in *Aspergillus spp.* (Beever and Laracy, 1986; Diano et al., submitted). Moreover the deletion of *gfdA* affects the carbon fluxes leading to an accumulation of organic acids and to an increase in the carbon dioxide yield, indicating an increased activity of the TCA cycle and/or the activity of the PP pathway as the specific production rate of erythritol increases. In parallel, less carbon is directed towards biomass production and the mitochondrial activity is increased as the oxygen consumption per glucose consumed increases.

During oxygen limitation, it becomes problematic for the cell to reoxidize NADH. As the wild type and the mutant have the same specific growth rates and the same specific glucose consumption rates, this indicates that G3PDH does not play an important role in NADH reoxidation at oxygen-limiting conditions. However, the metabolite quantification indicates a change in carbon flux repartition in the *gfdA* mutant and more especially it indicates an increase of 6-phosphofructokinase activity. Indeed, the activity of the pathway upstream the 6-phosphofructokinase seems to decrease in the *gfdA* mutant as the specific production rates of mannitol and erythritol decrease, while the TCA cycle activity seems to increase with an increase in carbon dioxide and organic acids specific production rates. This could be a consequence of an increased oxygen specific consumption rate. Indeed, the study of Fillinger et al. (2001) points out that a deletion of *gfdA* changes the chitin structure of the hyphae and therefore decreases the rigidity of the cell wall. In another study, Zetelaki and Vas (1970) observed a link between the rigidity of the cell wall and the medium viscosity. Therefore, a deletion of *gfdA* may cause a decrease in the medium viscosity, which may improve the oxygen transfer and this could explain the increase in the specific consumption rate of oxygen in the oxygen limitation phase of the batch fermentations. This variation in the micromorphology of the fungus may act in parallel with the fragmentation of the hyphae, which lowers the viscosity of the media.

### c. Influence of a double deletion of *mpdA* and *gfdA*

Figures 12 and 13 show the relative importance of *mpdA* and *gfdA* in exponential and oxygen limitation phases using the Principal Component Analysis tool (PCA). While the double mutant is grouped together with the *gfdA* mutant in the exponential growth phase (Figure 12), it is closer to the *mpdA* mutant in the oxygen limitation phase (Figure 13). This indicates that the double deletion mutant is more affected by the lack of G3PDH activity in the exponential growth phase and by the lack of MPD activity in the

oxygen limitation phase. Moreover, in figure 13, malic and fumaric acids are grouped together separately from the other organic acids. This suggests that, during oxygen limitation, malic and fumaric acids are produced by another pathway than pyruvic, citric and succinic acids. One hypothesis is that the production of pyruvic, citric and succinic acids is linked to the oxidative part of the TCA cycle, while malic and fumaric acids would be produced via oxaloacetate, allowing the reoxidation of NADH. This seems to be the main pathway for NADH reoxidation at oxygen limitations, when MPD activity is suppressed. Interestingly the deletion of both dehydrogenases did not lead to the production of acetate nor ethanol.

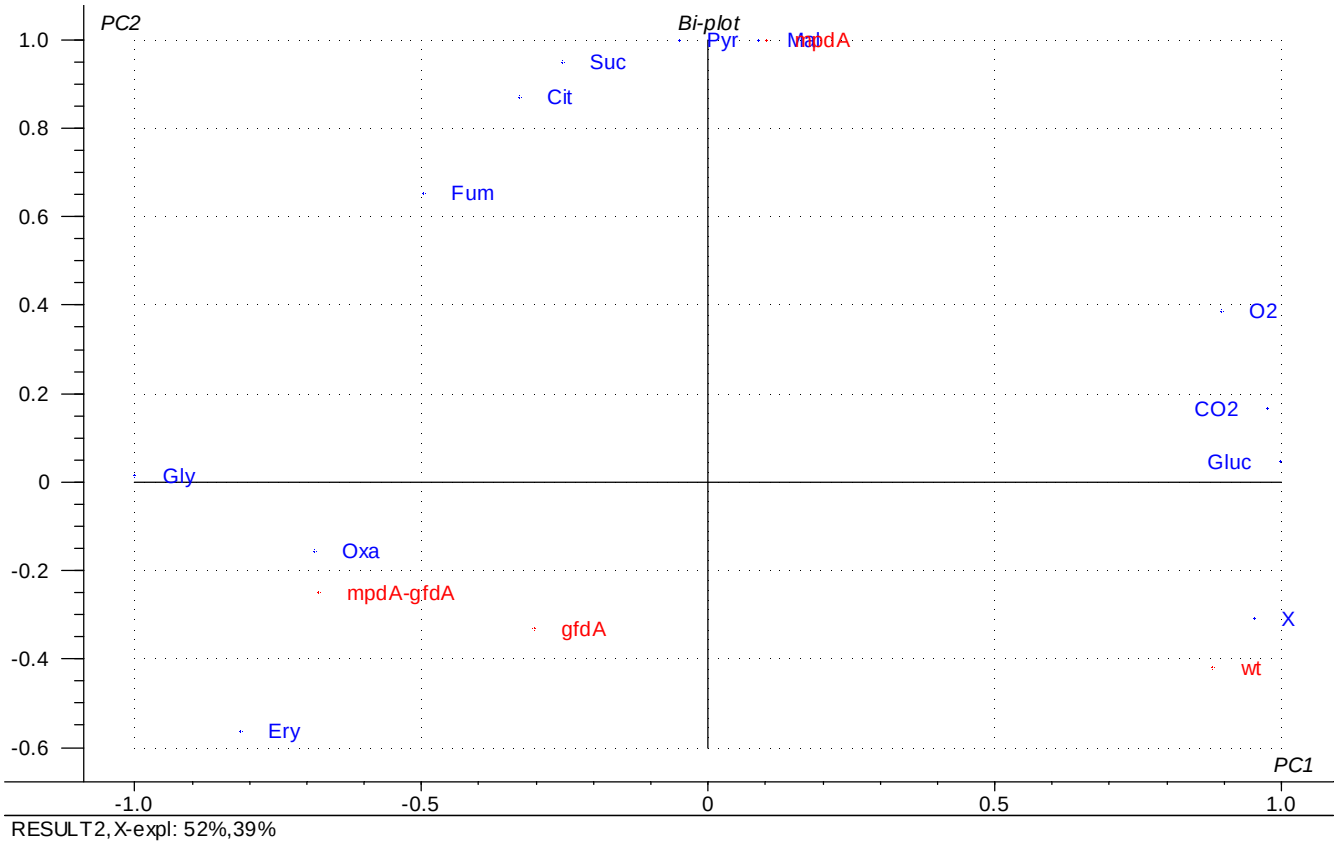


Figure 12 - PCA of the specific rates of consumption of oxygen (oxygen), glucose (gluc), and production of biomass (X), glycerol (gly), erythritol (ery), succinic acid (suc), fumaric acid (fum), pyruvic acid (pyr), malic acid (mal), oxalic acid (oxa) and CO2 (CO2), in Cμmol/gX/h, during the exponential phase of the wild type and mutant strains: *mpdA*, *gfdA* and double mutant *mpdA-gfdA*. This PCA has been made by "The unscrambler" program. The data sets have been first normalized by standardisation.

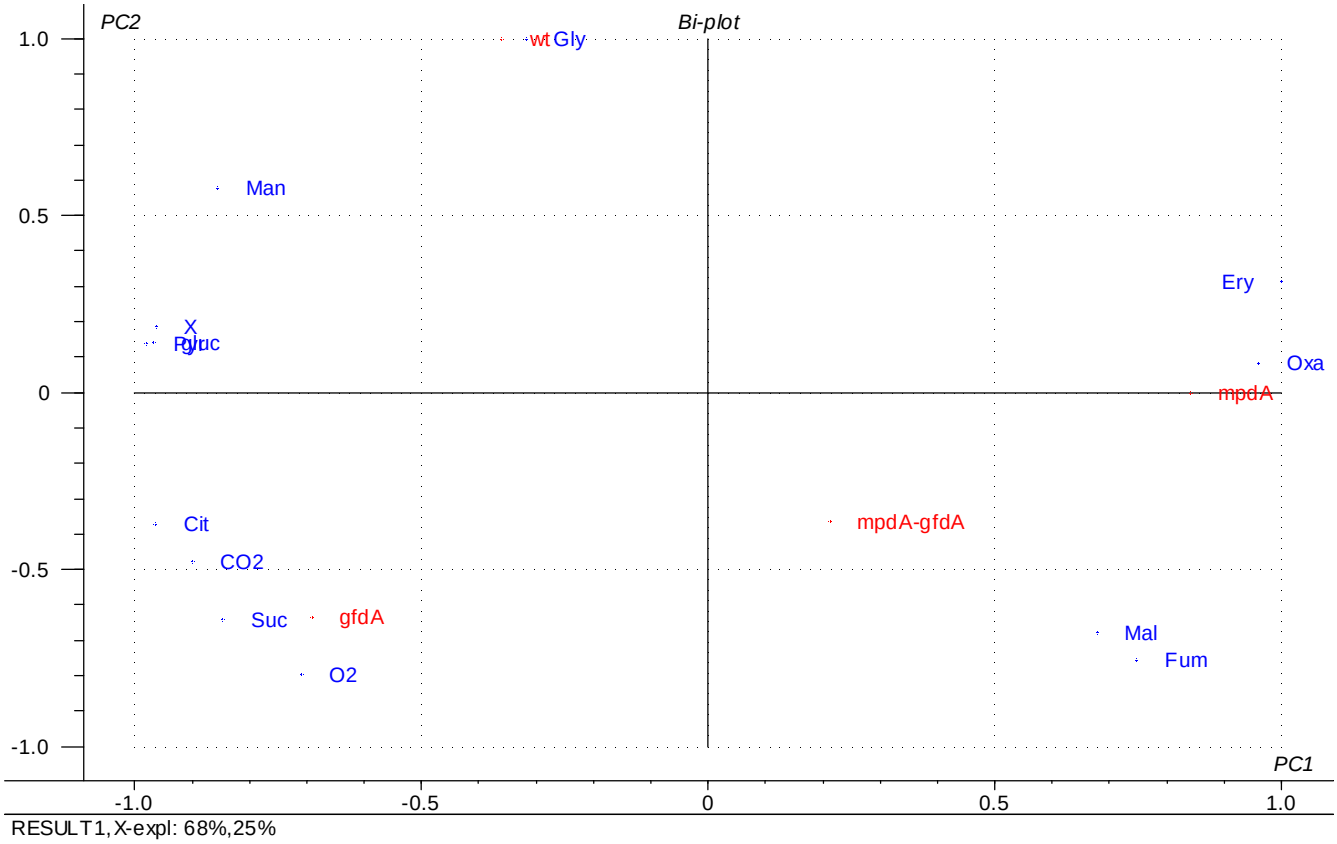


Figure 13 - PCA of the specific rates of consumption of oxygen (O2), glucose (gluc), and production of biomass (X), glycerol (gly), erythritol (ery), mannitol (man), succinic acid (suc), fumaric acid (fum), pyruvic acid (pyr), malic acid (mal), oxalic acid (oxa) and CO2 (CO2), in Cμmol/gX/h, during the oxygen limitation phase of the wild type and mutant strains: *mpdA*, *gfdA* and double mutant *mpdA-gfdA*. This PCA has been made by "The unscrambler" program. The data sets have been first normalized by standardisation.

### d. Role of *gfdB*

The *gfdB* gene was expressed neither in the wild type nor in the *gfdA* deleted mutants and its role remains unclear. GfdB is not involved in oxygen limitation and seems not to be involved in the response to high osmotic stress as, under these conditions, a deletion of *gfdA* led to a suppression of G3PDH activity (Fillinger et al., 2001). However, after deletion of *glcA*, encoding for GK (Figure 2), in a *gfdA* mutant, Fillinger et al. (2001) have observed the presence of G3P under hyperosmotic growth, indicating the presence of an yet uncharacterized enzyme. This enzyme could be G3PDH encoded by *gfdB* or GK encoded by *glcB* as two ORFs corresponding to this enzyme have also been detected in the genome of *A. nidulans* (David et al., 2006). The existence of a $FADH_2$ dependent G3PDH has also been mentioned by Hondmann (1994).

## 5. Conclusion

The study of the physiology and morphology of *mpdA* mutant as well as the MPD enzyme assays point to the important role of MPD during oxygen limitation and confirm the results of the previous chapters. However, this work demonstrates that *mpdA* is not an essential gene for the survival of *A. niger* and that a deletion of *mpdA* is to some extent compensate by the activation of other pathways like malate anabolic pathway, which can assist in reoxidising NADH. Thereby these results point to the metabolic rosbustness of *A. niger*. This work also underlines the importance of *mpdA* in non-oxygen-limited growth conditions, although no mannitol is detected in the medium at this condition. More precisely, this study suggests a role of the mannitol pathway in supplying fructose, which may have a role in the cell wall biosynthesis. Concerning *gfdA*, this work showed that G3PDH plays a minor role in reoxidising NADH at low oxygen availability. G3PDH, however, is still important for the growth of *A. niger* due to its possible link to the cell wall biosynthesis. Finally, it is interesting to notice the different responses of *A. niger* and *S. cerevisiae* to low levels of oxygen. *A. niger* reoxidises one mole NADH per mole glucose by producing mannitol, which is an important compound for the protection of spores against stress like high temperature and oxidation (Ruijter et al., 2003), for carbon storage (Witteveen and Visser, 1995) and during the germination process (Horikoshi et al., 1965; Witteveen and Visser, 1995). On the other hand, *S. cerevisiae* produces preferentially glycerol, a 3-carbon compound, hereby increasing the efficiency of the response since, in this case, two moles NADH can be reoxidised per mole glucose. This difference can be due to the fact that at low oxygen availability *Aspergillus* metabolism seems to be directed towards preparing for the sporulation process, whereas *S. cerevisiae*, which can grow under anaerobic conditions, applies instead a more energetically efficient NADH reoxidation system.

# *V.* Recombinant bacterial hemoglobin alters metabolism of *Aspergillus niger*

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*The results of this chapter will form the basis for a publication (in preparation).*

## 1. Abstract

Here the effects of the insertion of a bacterial hemoglobin gene on the metabolism of the filamentous fungus *Aspergillus niger* were studied. The *vgb* gene from *Vitroescilla sp.* was integrated into *A. niger* genome at the *pyrA* locus under the control of the strong *gpdA* promoter from *Aspergillus nidulans*. Interestingly, the analyses of secreted metabolites, oxygen uptake, carbon dioxide evolution, and biomass formation point towards a relief of stress in the VHB expressing mutant, when this strain is exposed to oxygen-limited conditions. Oxygen limitation is one of the challenges that have to be faced in cultivations of filamentous fungi, due to the high viscosity of the culture broth and the associated problems of obtaining a homogenous environment in the fermenter. Our findings therefore point to an interesting strategy to attenuate unwanted side effects resulting from oxygen limitation.

## 2. Introduction

*A. niger* is widely exploited as a cell factory in different biotechnological production processes. Its applications range from the production of pharmaceutical compounds and industrial enzymes to primary metabolites such as citric acid, and several production processes have been classified as "General Regarded As Safe" (GRAS) (Schuster et al., 2002).

However, one of the challenges often encountered in these production processes, is the high viscosity of the fermentation broth, particularly at high biomass concentration. This leads to several problems with respect to mixing and oxygen delivery and it is therefore valuable to identify new strategies that can improve the process performance by reducing for example the impact of oxygen depletion. One strategy that could possibly provide these results is the expression of an oxygen carrying protein in the host cell, as suggested previously (Khosla and Bailey, 1988). Subsequently it has been demonstrated that the expression of the *vgb* gene, which encodes a hemoglobin protein (VHB) from the bacteria *Vitreoscilla sp.*, improves many different biotechnological processes in a whole range of different prokaryotic and eukaryotic organisms (Frey and Kallio, 2003). Two recent examples are the production of beta-galactosidase by *Enterobacter aerogenes* (Khleifat et al., 2006), and the production of alpha-amylase by the yeast *Schwanniomyces occidentalis* (Suthar and Chattoo, 2006).

This study was therefore designed to study the impact of an intracellular expression of VHB on the metabolism of *A. niger* and to investigate if this approach of inverse metabolic engineering (Bailey et al. 2002) could also be fruitful for processes using filamentous fungi.

## 3. Material and Methods

### a. Strain, Media, Growth Conditions

The strains used in this study were *A. niger* A733 (also called N402) [*cspA1*] and *A. niger* A742 [*cspA1; pyrA5*], and both were obtained from the Fungal Genetics Stock Center. The media for plates and shake-flasks were prepared as described by Cove (1966).

The medium composition, fermentation set up and cultivation conditions for the batch cultivations have been previously described by Diano et al. (2006). However, in the present study the ammonium source used was 12 g/L of $NH_4Cl$, the carbon source was 110 g/L of monohydrate glucose, the working volume was 4.5 L and the stirring was set to 700 rpm. Briefly, the same number of spores of each strain was used for the inoculation of in-house built 5 L glass fermenters, and the aeration in the bioreactors was set to 0.2 vvm in order to achieve oxygen limitation after an initial exponential growth phase. The amount of oxygen during the fermentation was followed with an oxygen sensitive electrode and this was used to determine the onset of the oxygen-limited growth phase. Samples were taken in duplicate every 4 hours as described by Diano et al. (2006).

### b. Strain construction

Standard molecular techniques were carried out according to Sambrook et al. (2001). Genomic DNA was isolated from overnight shake-flask cultures (150 rpm, 30°C) with 100 mL of minimal medium supplemented with 2.44 g/L of uridine and 1.12 g/L of uracil. The mycelia were harvested by filtration, frozen in liquid nitrogen, crushed with a mortar and pestle, and approximately 200 mg were transferred to 750 µl of breaking buffer (50 mM Tris-HCl pH 7.5, 20 mM EDTA). After vortexing, 25 µl of 20% Sodium Dodecyl Sulfate was added, and the mixture was incubated at 65°C for 30 minutes. This was followed by the addition of 225 µl of 5 M potassium acetate and further incubation for 30 minutes on ice. After centrifugation for 10 minutes at 10,000 g the supernatant was transferred into a fresh tube, 2 volumes of 96% ethanol were added and followed by another centrifugation for 10 minutes at 15,000 g. The pellet was washed with 70% ethanol, the ethanol was removed, and the pellet was dried for 5 minutes at room temperature. The resulting DNA was dissolved in 200 µl of sterile distilled water.

Primers for the PCR amplification of the *pyrA* locus were designed (PpyrA_fw 5'-GGAACTGCCTTTGCAGGTGTGGC-3'; PpyrA_re 5'-CACCCTATAATAGCCTGCAGGATC-3') to identify the specific mutation that leads to the pyrimidine auxotrophy in this strain: the resulting PCR products from genomic DNA of the *A. niger* strains A733 and A742 were first subcloned into pGEM-T (Promega, USA), resulting in the plasmids pGEM-

T_pyrA and pGEM-T_pyrA5 respectively, and subsequent sequencing of the inserts was carried out by MWG Biotech (Germany).

For the construction of the plasmid pANvHb, the *vgb* gene was amplified by PCR from the plasmid pV6h, obtained from Dr. Bülow (Lund University, Sweden), using the primers Pvhb_NcoI_fw (5'-AGTACCCATGGATGTTAGACCAGCAAACCAT-3') and Pvhb_BamHI_re (5'-GCGGATCCTTATTCAACCGCTTGAGCG-3'). The resulting PCR fragment was digested with *Bam*HI and *Nco*I and cloned into the plasmid pMH-C (Bautista et al., 2000) from which the *creA* antisense fragment has been removed by digestion with *Bam*HI and *Nco*I and subsequently purified from an agarose gel. A *Eco*RI-*Not*I fragment of the *pyrA* gene (cut out from pGEM-T_pyrA), which can complement the pyrimidine auxotrophy, if it is integrated at the *pyrA5* locus, was blunt ended and ligated to the *Hind*III digested and blunt ended pANvHb to give the plasmid pANvHb_pyrA (Figure 1).

This plasmid was transformed into *A. niger* A742 by the method described by Nielsen et al. (2006), and in a parallel transformation the plasmid pGEM-T_pyrA was also transformed in the same strain. Transformants were recovered and selected on solid minimal medium.

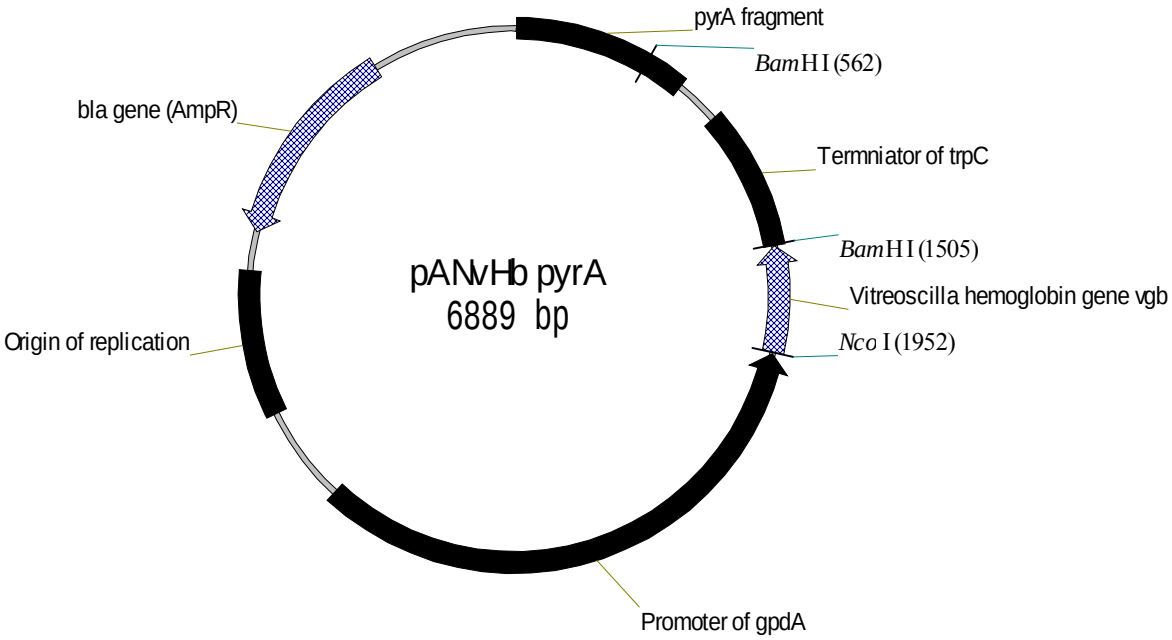


Figure 1 - Map of plasmid pANvHb_pyrA; for details of construction see Material and Methods.

### c. Metabolite analysis

Quantifications of substrate and products were carried out as detailed by Diano et al. (2006) but using 2 mM $H_2SO_4$ as eluent. Trehalose, xylitol and acetate were also meas-

ured, but data on these compounds will not be mentioned further, as no significant amounts were detected.

## 4. Results

### a. Strain construction

In order to integrate the *vgb* gene at a specific locus in the genome of *A. niger*, the mutation conferring the pyrimidine auxotrophy in the strain *A. niger* A742, *pyrA5*, was identified by sequencing the *pyrA* locus of that strain. This revealed a single C to T transition at position 751 (downstream of the translation initiation codon ATG) of the *pyrA* gene that gives rise to a stop codon instead of a glutamine, and thereby presumably resulting in a truncated and non-functional form of the orotidine-5-phosphate decarboxylase, whose functionality is required for the biosynthesis of pyrimidines.

The gene encoding the bacterial hemoglobin was cloned in a plasmid flanked by the promoter of the *gpdA* gene (Punt et al., 1991), which supposedly should result in constitutive expression of the bacterial hemoglobin, and the terminator of the *trpC* gene from *A. nidulans*. A fragment of the *pyrA* gene was also inserted into this plasmid, which was subsequently designated pANvHb_pyrA (Figure 1).

Transformation of *A. niger* A742 with this plasmid resulted in transformants that were no longer auxotrophic for pyrimidines due to the integration of the vector at the *pyrA* locus. These transformants also had the *vgb* gene integrated in the same locus, which was subsequently confirmed by PCR (data not shown). The hereby obtained strain was named *A. niger* VHB. The strain obtained after transformation with the plasmid pGEM-T_pyrA and subsequent selection on minimal medium was named *A. niger* $PYR^+$, and was used as reference strain throughout this study.

### b. Strain characterization

To investigate the impact of the insertion of the bacterial hemoglobin on the physiology of *A. niger*, batch fermentations were carried out with *A. niger* VHB and *A. niger* $PYR^+$.

During the exponential growth phase, which lasted approximately 13h for both strains, there was no significant difference between the maximum specific growth rates for the two strains equivalent to 0.248 $h^{-1}$ ± 0.016. The carbon was mainly oriented to the production of biomass and carbon dioxide. Glycerol was the main metabolite produced in concentrations up to 1 g/L.

Approximately 31 hours after inoculation the dissolved oxygen tension dropped below 1% for both strains and the cells entered in a linear growth phase. At this point, the secretion of significant amounts of glycerol, erythritol, mannitol, arabitol and ethanol

was observed, while very low amounts of organic acids were produced (Figure 2 and Table 1).

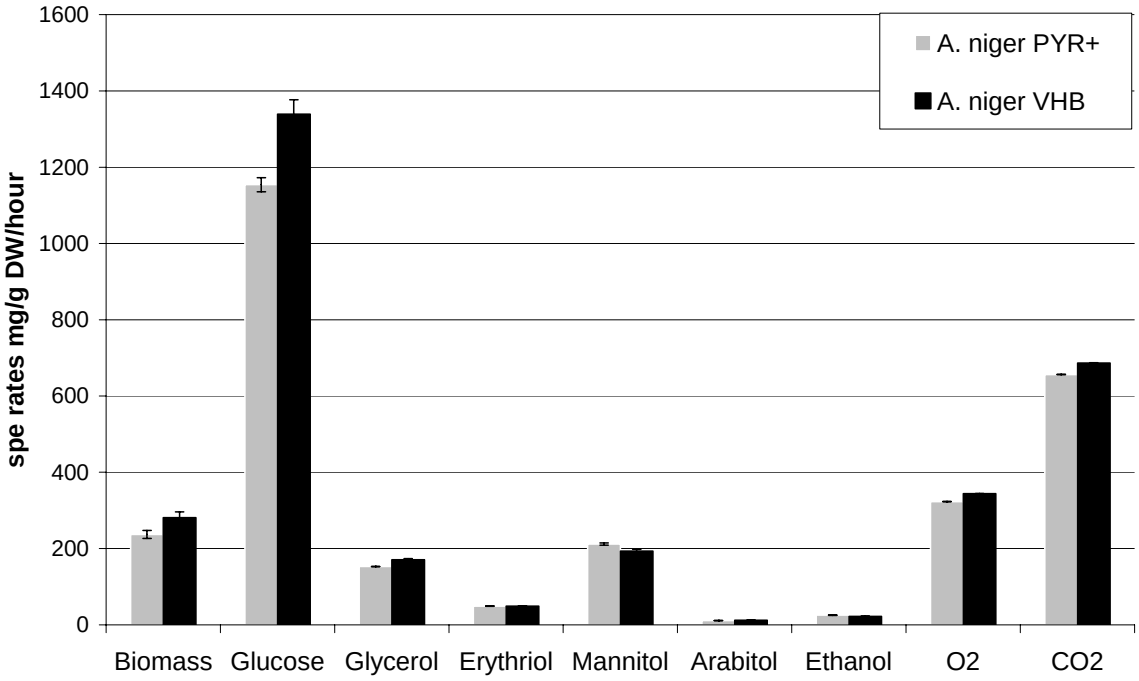


Figure 2 - Specific consumption and production rates of *A. niger* PYR+ (grey bars) and *A. niger* VHB (black bars) during oxygen-limited growth conditions; the values were calculated as average of duplicate samples and represent the overall rates of metabolites divided by the average biomass concentration during the oxygen limition phase. Standard deviations are indicated by error bars.

Table 1 – Yields and carbon balance of *A. niger* PYR+ and *A. niger* VHB during oxygen-limited growth conditions. Values and standard deviations were calculated as average of duplicate samples.

| | *A. niger* PYR+ | | *A. niger* VHB | |
|---|---|---|---|---|
| | AVE | STD | AVE | STD |
| Biomass | 0.247 | 0.007 | 0.253 | 0.007 |
| Glycerol | 0.129 | 0.002 | 0.125 | 0.006 |
| Erythriol | 0.042 | 0.001 | 0.036 | 0.001 |
| Mannitol | 0.181 | 0.005 | 0.143 | 0.007 |
| Arabitol | 0.009 | 0.000 | 0.009 | 0.000 |
| Succinate | 0.001 | 0.000 | 0.002 | 0.001 |
| Fumarate | 0.003 | 0.000 | 0.002 | 0.000 |
| Citrate | 0.005 | 0.000 | 0.002 | 0.000 |
| Pyruvate | 0.002 | 0.000 | 0.000 | 0.001 |
| Oxalate | 0.001 | 0.000 | 0.000 | 0.000 |
| Malate | 0.004 | 0.000 | 0.003 | 0.000 |
| Ethanol | 0.029 | 0.001 | 0.022 | 0.002 |
| $O_2$ | 0.263 | 0.004 | 0.241 | 0.006 |
| $CO_2$ | 0.388 | 0.007 | 0.349 | 0.009 |
| C balance | 1.041 | 0.009 | 0.945 | 0.020 |

During the course of the oxygen-limited growth phase, a number of differences could be observed between the *vgb* expressing strain and the reference strain. The average specific growth rate of the *vgb* strain was determined to be 0.016 $h^{-1}$ ± 0.000, whereas the reference strain grew at 0.013 $h^{-1}$ ± 0.001. This reflects a significant higher growth for the strain that carries the bacterial hemoglobin. Interestingly, the same trend was also found for the specific glucose uptake rate, which shows a 19% increase (Figure 2). Furthermore offgas analysis revealed that the specific oxygen uptake rate was increased in the *vgb* strain, but only slightly (less than 10%), and this was also found for the carbon dioxide production rate (Figure 2).
Finally the yields presented on figure 3 show that the main consequence associated to the insertion of *vgb* was a reduction of the carbon flux in the mannitol and ethanol pathways up to 24%.

## 5. Discussion

Since it has been described previously that the recombinant expression of *Vitreoscilla* hemoglobin in microbes evokes different effects in the metabolism of the host cell depending on the amount of available oxygen, the cultivation strategy chosen in this study was to restrict the air flow to 0.2 vvm, resulting in an initial exponential growth phase during which oxygen is not limiting, and a subsequent growth phase defined by oxygen-limited growth.
The analysis of all the measured parameters for the cultivations showed that during the oxygen non-limited phase there were no or only very small differences between the strain that harbors the recombinant hemoglobin and the reference strain. This therefore suggests that *A. niger* does not undergo any major physiological changes due to the genomic insertion and expression of the *vgb* gene under oxygen non-limited growth conditions. The data obtained in this study also show that glycerol was the only polyol secreted in significant amounts during oxygen non-limited growth, independently of the insertion of the bacterial hemoglobin. This confirms previously obtained results (Diano et al., 2006), and is most likely a result of the high osmotic, due to the high sugar concentration in the medium, as the accumulation of glycerol has already previously been linked to osmotic stress (de Vries et al., 2003; Fillinger et al., 2001) .

However, like *S. cerevisiae* (Chen et al., 1994), *Yarrowia lipolytica* (Bhave and Chattoo, 2003), *E. coli* (Tsai et al., 1996), and other microorganisms, *A. niger* VHB shows significant changes in its physiology during oxygen-limited conditions when compared to the reference strain.

One effect which is consistent with the observation in the above mentioned organisms is the increase in the specific growth rate. In this study, we find an approximately 16% increase in the specific average growth rate in the strain harboring the bacterial hemoglobin gene during the oxygen-limited growth phase. However there is no significant increase in the biomass yield, which is a result of the increased glucose uptake rate of *A. niger* VHB.

Analysis of the formation rates of metabolites that were secreted during the cultivations of the two *A. niger* strains allowed a quite detailed insight into the metabolic changes that occurred in the *vgb* harboring strain.

Since *A. niger* is well known for its outstanding production capabilities for organic acids, it was investigated in detail if the introduction of the recombinant hemoglobin gene exhibits any effects on the TCA cycle, although low amounts of organics acids were produced under the present cultivation conditions. The general decrease of the TCA cycle intermediates specific yields observed here in the *vgb* mutant has also been mentioned in a similar study using *E. coli* (Tsai et al. 1996). This reduction of the organic acid formation rates supports the hypothesis that the expression of VHB enables the cell to work more efficiently during oxygen limitation, since it has been described previously that oxygen limitation normally results in an increase of excretion of TCA cycle compounds (Diano et al., submitted).

Another interesting group of compounds that was investigated in detail in this study is the polyols, since their metabolism involves the transfer of reduction equivalents, and this therefore links them to the respiration of the cell. Previous studies of *A. niger* under oxygen limitation, which used a nearly identical experimental setup, but a different strain background (*A. niger* BO1), have shown that mannitol levels are directly connected to the availability of oxygen (Diano et al., submitted; Diano et al., 2006). An explanation for the accumulation of mannitol when oxygen becomes limiting is that the biosynthesis of this polyol is a way for the organism to reoxidise NADH to $NAD^+$ by utilizing the MPD, which has been shown to be involved in oxidative and other stress responses in *A. niger* (Ruijter et al., 2003). Here we report that the expression of the *Vitreoscilla* hemoglobin in *A. niger* reduces the mannitol yield by approximately 21% under oxygen-limited conditions. This could be an indication that the VHB interacts with the respiratory chain, assists with the reoxidation of NADH, and thereby reduces the accumulation of mannitol. This is further supported by our finding that *vgb* expression also significantly reduces the ethanol yield, which has also been directly linked to alterations of the respiratory chain by Bradshaw et al. (2001), who reported an increasing production of ethanol after deletion of the *cycA* gene (encoding for cytochrome C).

An increase of the formation rate of glycerol under oxygen-limiting conditions in *A. niger* VHB was also found but this was associated to an increase in the glucose consumption rate leaving the glycerol yield unchanged.

Another indication of the interaction of VHB with the respiratory chain is the increase in oxygen uptake rate in *A. niger* VHB. This observation has been previously reported for cells harboring the *vgb* gene (Chen et al., 1994; Khosla and Bailey, 1988; Suthar and Chattoo, 2006). However, other studies have also reported the opposite effect (Wei et al., 1998). This increase in the flux of oxygen through the cell might be a direct effect of the ability of VHB to bind oxygen (Wakabayashi et al., 1986), and is another indication that VHB has an effect on the respiration and thereby on the redox metabolism of *A. niger*. However, since our results indicate that there is only a small increase in the specific oxygen consumption rate (7%), this suggests that the major effects of VHB within the *A. niger* cell are not due to its capability of capturing additional oxygen.

Another function of the bacterial hemoglobin during oxygen limitation could be a role as oxygen storage in the cells. Indeed rather than improving the overall uptake of oxygen, the hemoglobin might just act as a storage buffer that reduces the impact of temporary oxygen deprivation spikes, which occur when the mycelium travels through oxygen depleted zones. The importance of these zones in high viscous filamentous fungi broth has been previously studied by Manfredini et al. (1983). This hemoglobin function could explain the relatively small increase in the oxygen consumption rate associated to a significant stress relief in oxygen limitation.

## 6. Conclusion

This study shows that the expression of the bacterial hemoglobin gene from *Vitreoscilla* has a relatively strong impact on the metabolism of *A. niger* under oxygen-limited conditions. It appears that the presence of VHB leads to a relief of the stress experienced by the cells when oxygen is only available in small amounts. This is reflected by an increase in the average specific growth rate, and an overall reduction of the yields for the formations of organic acids, ethanol and polyols.

The mechanisms through which VHB accomplishes these alterations of the metabolism is not clear, but the results indicate that VHB interferes in the redox metabolism of the cell, and assists the reoxidation of NADH. This could be attributed to one of several hypotheses about the intracellular roles of VHB, such as its potential for binding oxygen or its function as a terminal oxidase.

Overall, this study clearly shows that the expression of VHB in *A. niger* has advantageous effects on its physiology under oxygen-limited conditions and this strategy could

be applied to filamentous fungi processes where the oxygen transfer is reduced by the high broth viscosity.

# VI. Conclusions and future perspectives

This study dealing with the physiology of *A. niger* in oxygen limitation led naturally to the study of the roles of polyols, as polyols are one of the most important group of compound in fungal mycelia (Blumenthal, 1976). Although the production of all polyols involved the reoxidation of the cofactor NADH or NADPH and could potentially be involved in the metabolic answer to oxygen limitation, the production of only one polyol, mannitol, has shown to be directly linked to oxygen limitation. The over production of mannitol requires also low growth rates, which may be linked to an increase in mannitol production at these conditions and to a fast reconsumption of mannitol at high growth rates. Indeed, this work showed the importance of the mannitol pathway in non-oxygen limited conditions even though, at these conditions, no mannitol is accumulated, which indicates a successive production and reconsumption of this compound. This work has also shown a link between the MPD and the activity of the TCA cycle, as the deletion of this enzyme increases the production of all TCA cycle intermediates. In parallel, this work has pointed out the limited role of the NAD-dependent glycerol-3-phosphate dehydrogenase in oxygen limitation and by that, this study has pointed out a main difference between *A. niger* and *S. cerevisiae* metabolism. In *Aspergillus spp.* the NAD-dependent glycerol-3-phosphate dehydrogenase is involved in cell wall synthesis and glycerol as well as erythritol and xylitol are mainly involved as osmoregulator compounds. Moreover this work has shown a leakage of osmoregulators through the membrane, which decreases the efficiency of the osmotic stress response. A role of polyols as carbon storage compounds has also been demonstrated, particularly concerning polyols from the PP pathway such as erythritol and xylitol.

All this information allowed drawing a picture of the events happening in typical industrial fermentation using *A. niger* (Figure 1). The high glucose concentration leads to a high osmotic pressure and hence to the production of osmoregulator compounds like glycerol, erythritol and xylitol, and furthermore to high production of biomass, which increases the viscosity of the medium and decreases the oxygen availability. When a period of oxygen limitation is reached, the decrease in the mitochondria activity leads to a decrease in ATP production and NADH reoxydation. A direct consequence is a diminution of growth rate and an overflow at the PP level leading mainly to the production of erythritol. The increase of the catabolic reduction of charge associated to low growth rate leads to the excretion of mannitol.

This study has focused on the physiology of an industrial strain *A. niger BO1*. In the last chapter, the use of strains descending from *A. niger N402* shows a similar behav-

iour with production of mannitol at oxygen limitation, validating the role of mannitol in oxygen limitation for different *A. niger* strains. It is interesting to notice the absence of ethanol production in *A. niger BO1* fermentations, while ethanol is produced by *A. niger N402* under oxygen limitation.

While the first four chapters answer to the main question of this pHD. project "what are the consequences of a period of oxygen limitation on *A. niger* metabolism", the fifth chapter goes one step further by analysing one possibility to improve the efficiency of *A. niger* cell factory under oxygen limitation. In this last chapter, the insertion of the hemoglobin gene *vgb* from the bacteria *Vitreoscilla*, was shown to decrease the stress imposed by oxygen limitation, i.e. to increase glucose consumption and biomass production, while decreasing mannitol and ethanol production.

There are other strategies, which could limit the consequences of a period of oxygen limitation on the metabolism of *A. niger*. Among them, the introduction of a transhydrogenase, which could convert NADH and NADP into NAD and NADPH, could limit the intracellular accumulation of NADH, when oxygen is limiting. However, the introduction of transhydrogenase from different species into *S. cerevisiae* led to the inverse reaction depleting the NADPH pool and reducing the specific growth rate without implementing the growth in anaerobic conditions (Anderlund et al., 1999; Nissen et al., 2000a). It should be noticed that an endogenous *A. niger* transhydrogenase has recently been characterised by the DOE Joint Genome institute (http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Aspni1&tid=138792) and it would be interesting to study the expression of this gene particularly under oxygen limitation. Another possibility, to reduce the intracellular level of NADH, could be the integration of a water-forming NADH oxidase. This enzyme converts NADH into NAD using a molecule of oxygen as final acceptor. This strategy has been successful in *S. cerevisiae* (Heux et al., 2006b; Heux et al., 2006a) however in our particular case, the introduction of such an enzyme may compete for oxygen against the phosphorylative respiration and may therefore reduce the formation of ATP. A more suitable strategy would be the replacement of NADPH consuming reactions with NADH consuming reactions. This could be done at the level of the ammonium assimilatory pathway. Indeed, ammonium is assimilated into glutamate by reaction with 2-oxoglutarate and the enzyme catalysing this reaction is the NADPH-dependent glutamate dehydrogenase (Moye et al., 1985). However in some fungi like *S. cerevisiae* the existence of another glutamate synthesis pathway has been shown (Cogoni et al., 1995; Mitchell and Magasanik, 1983). This involved the NADH-dependent glutamate synthase and the glutamine synthetase leading to the following reaction:

2-oxoglutarate + $NH_4^+$ +NADH + ATP → glutamate + NAD + ADP + Pi

The overexpression of these both enzymes combined with the deletion of the NADPH-dependent glutamate dehydrogenase in *S. cerevisiae* has led to a reduction of glycerol

synthesis, which is produced by *S. cerevisiae* cells in order to reoxidize NADH (Nissen et al., 2000b). The same genetic modifications could be applied to *A. niger*, which could potentially reduce the intracellular accumulation of NADH in oxygen limitation and decrease the production of mannitol. Another strategy could be combined with the previous one by using nitrate as nitrogen source. Indeed the assimilation of nitrate implies the reduction of nitrate to ammonium. In *Aspergillus spp.*, this is done by the NADPH-dependent enzymes, nitrate reductase and nitrite reductase (Hankinson and Cove, 1974), while in other species, like *Fusarium oxysporum*, these both enzymes are NADH-dependent (Shoun and Tanimoto, 1991). In such organisms the use of nitrate instead of ammonium under anaerobic conditions increases the growth rate and decreases the acetate production (Panagiotou et al., 2006). Therefore the conversion of *A. niger* NADPH-dependent nitrate and nitrite reductases into NADH-dependent reductases could potentially lower the stress caused by oxygen limitation in cultures supplied by nitrate.

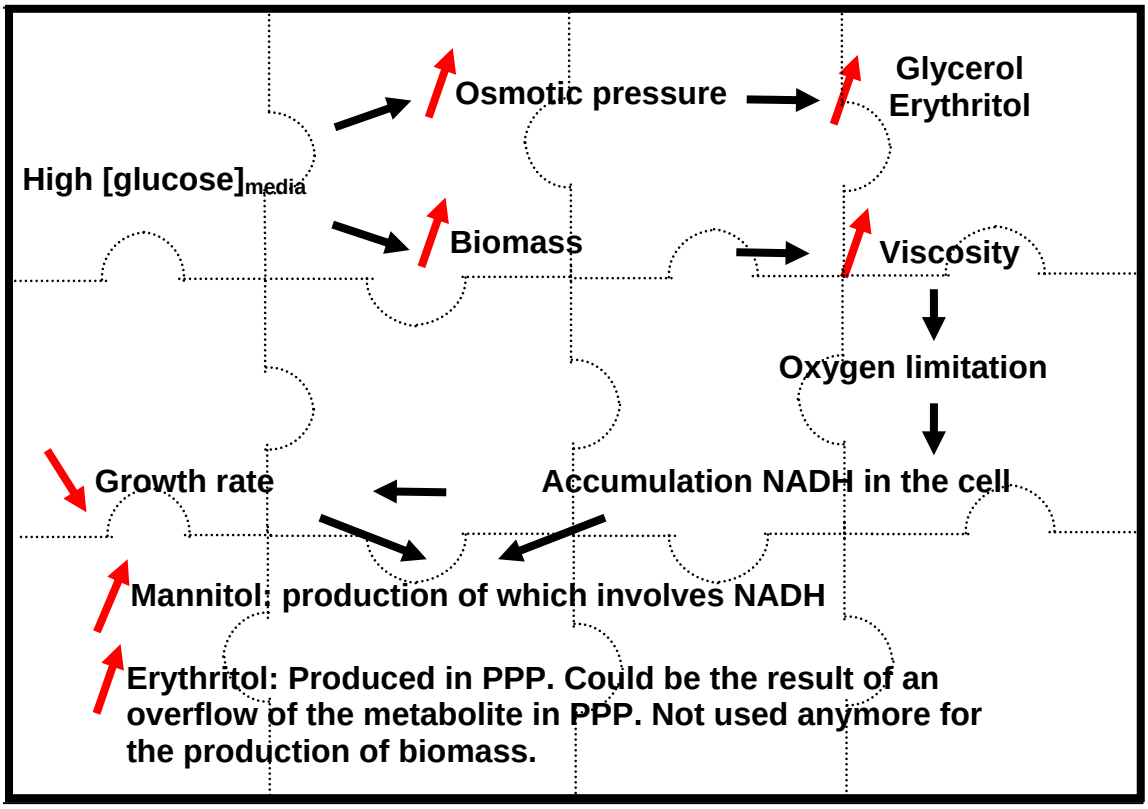


Figure 1 - Metabolic puzzle completed, this figure resumed the main events happening in high density batch cultivations.

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# VIII. Appendix

## 1. Appendix I

Tblastn of *A. nidulans* GfdA amino acid sequence on *A. niger* sequence 8.9x coverage.

```
TBLASTN 2.2.10 [Oct-19-2004]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query= L:\GENETI~1\AN0351~1.TXT
         (417 letters)

Database: C:\Program Files\Bioedit\database\an.fasta
           143 sequences; 37,196,396 total letters
                                                                    Score    E
Sequences producing significant alignments:                         (bits) Value

scaffold_1                                                              704   0.0
scaffold_6                                                              402   e-114

>scaffold_1
          Length = 3970925

 Score =  704 bits (1816), Expect(2) = 0.0
 Identities = 352/425 (82%), Positives = 376/425 (88%), Gaps = 25/425 (5%)
 Frame = -3

Query: 1       MGSLGPYKQKHKVTVVGSGNW------------------------GTAIAKIVAENTAS 35
                MGSLGPY++KHK+TVVGSGNW                      G AIAKIVAEN AS
Sbjct: 2177643 MGSLGPYQRKHKITVVGSGNWYVQAGFPSWC*CQGRRKRLTRVMYRGCAIAKIVAENAAS 2177464

Query: 36      NPAVFEKDVQMWVFEEKVEIPKSSKHYDPASSLCQGPQNLTDIINHTHENIKYLPGITLP 95
                NPA+FE+ V+MWVFEEKVEI K S+HYDP+S LCQGPQNLTD+IN  HENIKYLPGITLP
Sbjct: 2177463 NPAIFEEKVEMWVFEEKVEISKDSRHYDPSSPLCQGPQNLTDVINQKHENIKYLPGITLP 2177284

Query: 96      ENLIANPSLVDAVQDSTILVFNLPHQFIINICEQIKGKIVPYARGISCIKGVDVNEEGVH 155
                 NL ANPSLVDAV+DSTILVFNLPHQFII  CEQIKGKI+PYARGISCIKGVDVNEEG+H
Sbjct: 2177283 SNLHANPSLVDAVKDSTILVFNLPHQFIIKTCEQIKGKILPYARGISCIKGVDVNEEGIH 2177104

Query: 156     LFSETIGKILGIYCGALSGANIANEVAQEKWSESSIGYDPPHFDSKAPSPPNRSPSASTD 215
                LFSETIGKILGIYCGALSGANIA+EVA EKWSESSI YDPPH DSKAPSP NRSPS ST+
Sbjct: 2177103 LFSETIGKILGIYCGALSGANIASEVALEKWSESSIAYDPPHMDSKAPSP-NRSPSTSTE 2176927

Query: 216     NILHFEHKDVSGQLSRVKLQALPSEFPPIDHALLKSLFHRPYFHIGVVSDVAGVSLGGAL 275
                N++ FEHKDVSGQLSRVKLQALPS++PP+DHA+LKSLFHRPYFHI VVSDVAGVSLGGAL
Sbjct: 2176926 NLVQFEHKDVSGQLSRVKLQALPSDYPPVDHAVLKSLFHRPYFHIRVVSDVAGVSLGGAL 2176747

Query: 276     KNVVAVAAGWVVGKGWGDNAKAAIMRVGLLEMVKFGEQFFGATINTRTFTEESAGVADLI 335
                KNVVA+AAGWV G GWGDNAKAAIMRVGLLEMVKFGE+FFGATINTRTFTEESAGVADLI
Sbjct: 2176746 KNVVALAAGWVDGMGWGDNAKAAIMRVGLLEMVKFGEKFFGATINTRTFTEESAGVADLI 2176567

Query: 336     TSCSGGRNFRCAKLSIERNQPIEKIEETELNGQKLQGTLTAVEVNSFLKKQGLEEEFPLF 395
                TSCSGGRNFRCAKLSIER Q IEK+EETELNGQKLQGTLTAVEVN+FLKKQGLEEEFPL
Sbjct: 2176566 TSCSGGRNFRCAKLSIERKQSIEKVEETELNGQKLQGTLTAVEVNNFLKKQGLEEEFPLL 2176387

Query: 396     TAVYR 400
                TAVYR
Sbjct: 2176386 TAVYR 2176372
```

```
 Score = 28.9 bits (63), Expect(2) = 0.0
 Identities = 12/17 (70%), Positives = 16/17 (94%)
 Frame = -1

Query: 401     VLQGTMSVDEIPSFIER 417
               VL+G+MSV +IPS+IER
Sbjct: 2176304 VLEGSMSVGDIPSYIER 2176254


>scaffold_6
          Length = 2024198

 Score =  402 bits (1034), Expect(2) = e-114
 Identities = 210/378 (55%), Positives = 272/378 (71%)
 Frame = -1

Query: 22      GTAIAKIVAENTASNPAVFEKDVQMWVFEEKVEIPKSSKHYDPASSLCQGPQNLTDIINH 81
               G+ I+KIVAEN   +  +FE +V+MWVFEE++EIP+SSKH+   S L      LT++IN
Sbjct: 1865627 GSTISKIVAENAREHSDLFEPEVRMWVFEEEIEIPESSKHH---SKLGGQKHKLTEVING 1865457

Query: 82      THENIKYLPGITLPENLIANPSLVDAVQDSTILVFNLPHQFIINICEQIKGKIVPYARGI 141
                HEN+KYLP I LPEN++A+P L  AV+D+T+LVFNLPHQFI    + + G  +PYARGI
Sbjct: 1865456 VHENVKYLPDIALPENVVADPDLKSAVKDATLLVFNLPHQFIGKTLDGVVGHHLPYARGI 1865277

Query: 142     SCIKGVDVNEEGVHLFSETIGKILGIYCGALSGANIANEVAQEKWSESSIGYDPPHFDSK 201
               SCIKGVDV++  V L SE I + L IYCGALSGANIA EVA EK+ E++IGYD P  D +
Sbjct: 1865276 SCIKGVDVSDGTVTLHSELIMERLSIYCGALSGANIAPEVAAEKFCETTIGYDTPPMDVE 1865097

Query: 202     APSPPNRSPSASTDNILHFEHKDVSGQLSRVKLQALPSEFPPIDHALLKSLFHRPYFHIG 261
                     R  S   + I   E +    + + VKL  +P + P +D  L ++LF RPYFH+
Sbjct: 1865096 -----ERDGSPKENLIKIDEQRQHKTKPTHVKLHPVPKDLPAVDAELWETLFARPYFHVN 1864932

Query: 262     VVSDVAGVSLGGALKNVVAVAAGWVVGKGWGDNAKAAIMRVGLLEMVKFGEQFFGATINT 321
                V DVAGV+LGGALKN+VA+A+G+V GKGWG+NAKAAIMRVG+LEM+KFG  +F  +++
Sbjct: 1864931 HVRDVAGVALGGALKNIVALASGFVAGKGWGENAKAAIMRVGILEMIKFGRTWFPKSVDE 1864752

Query: 322     RTFTEESAGVADLITSCSGGRNFRCAKLSIERNQPIEKIEETELNGQKLQGTLTAVEVNS 381
               RTFTEESAG+AD+I+SCSGGRNFR A  ++E+   + +IE+ ELNGQKLQGT TA  V
Sbjct: 1864751 RTFTEESAGLADVISSCSGGRNFRSACHAVEQGVSVNEIEQKELNGQKLQGTSTAYAVYD 1864572

Query: 382     FLKKQGLEEEFPLFTAVY 399
               FL K    +EFPLF AV+
Sbjct: 1864571 FLSKHDQLKEFPLFVAVH 1864518



 Score = 29.6 bits (65), Expect(2) = e-114
 Identities = 11/15 (73%), Positives = 13/15 (86%)
 Frame = -2

Query: 7       YKQKHKVTVVGSGNW 21
               + +KHKV VVGSGNW
Sbjct: 1865731 HTRKHKVAVVGSGNW 1865687


  Database: C:\Program Files\Bioedit\database\an.fasta
    Posted date:  Jul 26, 2006  4:44 PM
  Number of letters in database: 37,196,396
  Number of sequences in database:  143

Lambda     K      H
   0.316    0.134    0.396

Gapped
Lambda     K      H
```

```
   0.267   0.0410    0.140


Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Hits to DB: 18,828,140
Number of Sequences: 143
Number of extensions: 308749
Number of successful extensions: 2092
Number of sequences better than  1.0: 2
Number of HSP's better than  1.0 without gapping: 14
Number of HSP's successfully gapped in prelim test: 10
Number of HSP's that attempted gapping in prelim test: 1622
Number of HSP's gapped (non-prelim): 1109
length of query: 417
length of database: 12,398,798
effective HSP length: 104
effective length of query: 313
effective length of database: 12,383,926
effective search space: 3876168838
effective search space used: 3876168838
frameshift window, decay const: 40,  0.1
T: 13
A: 40
X1: 16 ( 7.3 bits)
X2: 38 (14.6 bits)
X3: 64 (24.7 bits)
S1: 41 (21.6 bits)
S2: 71 (32.0 bits)
```

## 2. Appendix II

Tblastn of *A. nidulans gfdb* translated sequence on *A. niger* sequence 8.9x coverage.

```
TBLASTN 2.2.10 [Oct-19-2004]


Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query= L:\GENETI~1\AN6792~1.TXT
         (407 letters)

Database: C:\Program Files\Bioedit\database\an.fasta
           143 sequences; 37,196,396 total letters
                                                                    Score    E
Sequences producing significant alignments:                        (bits) Value

scaffold_6                                                              532   e-157
scaffold_1                                                              413   e-115
scaffold_8                                                               35   0.15

>scaffold_6
          Length = 2024198

 Score =  532 bits (1371), Expect(3) = e-157
 Identities = 259/370 (70%), Positives = 302/370 (81%)
 Frame = -1

Query: 18      GSTIGKILAENTSEHTDTFETPVRMWVFEEEITVPSDSPHHSKYGDKPQKLTQIINETHE 77
                GSTI KI+AEN  EH+D FE  VRMWVFEEEI +P  S HHSK G +  KLT++IN  HE
Sbjct: 1865627 GSTISKIVAENAREHSDLFEPEVRMWVFEEEIEIPESSKHHSKLGGQKHKLTEVINGVHE 1865448

Query: 78      NVKYLPGIKLPDNLIATPDIKEAVKDASILVFNLPHQFIGKTLDQIKGHHLPYARGVSCI 137
                NVKYLP I LP+N++A PD+K AVKDA++LVFNLPHQFIGKTLD + GHHLPYARG+SCI
Sbjct: 1865447 NVKYLPDIALPENVVADPDLKSAVKDATLLVFNLPHQFIGKTLDGVVGHHLPYARGISCI 1865268

Query: 138     KGVDVSDGKVTLFSELIMQKLGIYCGALSGANIAPEVAAERLCETTIGYDPPPMDLTSSD 197
                KGVDVSDG VTL SELIM++L IYCGALSGANIAPEVAAE+ CETTIGYD PPMD+   D
Sbjct: 1865267 KGVDVSDGTVTLHSELIMERLSIYCGALSGANIAPEVAAEKFCETTIGYDTPPMDVEERD 1865088

Query: 198     GSPEDNLPHVDGQRQKDLSSTDIKLERVPQDYPHVNDELLEKMFERPYFLVHVIKDVAGV 257
                GSP++NL  +D QRQ     T +KL  VP+D P V+ EL E +F RPYF V+ ++DVAGV
Sbjct: 1865087 GSPKENLIKIDEQRQHKTKPTHVKLHPVPKDLPAVDAELWETLFARPYFHVNHVRDVAGV 1864908

Query: 258     ALGGALKNIVALAAGFVAGKQWGENTKAAIIRQGVGEMIRFGRTWFPSSVNEKTFTEESA 317
                ALGGALKNIVALA+GFVAGK WGEN KAAI+R G+ EMI+FGRTWFP SV+E+TFTEESA
Sbjct: 1864907 ALGGALKNIVALASGFVAGKGWGENAKAAIMRVGILEMIKFGRTWFPKSVDERTFTEESA 1864728

Query: 318     GIADLVASCYGGRNVRSATHAVEKGVSVAEIEKTEMNGQKLQGVSTAQTVYGFLEKHGKT 377
                G+AD+++SC GGRN RSA HAVE+GVSV EIE+ E+NGQKLQG STA  VY FL KH +
Sbjct: 1864727 GLADVISSCSGGRNFRSACHAVEQGVSVNEIEQKELNGQKLQGTSTAYAVYDFLSKHDQL 1864548

Query: 378     DEFPLFCAVY 387
                 EFPLF AV+
Sbjct: 1864547 KEFPLFVAVH 1864518



 Score = 33.1 bits (74), Expect(3) = e-157
 Identities = 14/20 (70%), Positives = 18/20 (90%),
 Frame = -3

Query: 388     NIIEGKAQVDDLPALLNRKK 407
                +I+EGK+ VDDLPALL+ KK
```

```
Sbjct: 1864461 DILEGKSTVDDLPALLDGKK 1864402



 Score = 30.0 bits (66), Expect(3) = e-157
 Identities = 10/14 (71%), Positives = 14/14 (100%)
 Frame = -2

Query: 4       TKKHQVSVIGSGNW 17
                T+KH+V+V+GSGNW
Sbjct: 1865728 TRKHKVAVVGSGNW 1865687


>scaffold_1
          Length = 3970925

 Score =  413 bits (1061), Expect = e-115
 Identities = 215/415 (51%), Positives = 283/415 (68%), Gaps = 32/415 (7%)
 Frame = -3

Query: 5       KKHQVSVIGSGNW------------------------GSTIGKILAENTSEHTDTFETP 39
                +KH+++V+GSGNW                          G  I KI+AEN + +   FE
Sbjct: 2177619 RKHKITVVGSGNWYVQAGFPSWC*CQGRRKRLTRVMYRGCAIAKIVAENAASNPAIFEEK 2177440

Query: 40      VRMWVFEEEITVPSDSPHH---SKYGDKPQKLTQIINETHENVKYLPGIKLPDNLIATPD 96
                V MWVFEE++ +  DS H+   S     PQ LT +IN+ HEN+KYLPGI LP NL A P
Sbjct: 2177439 VEMWVFEEKVEISKDSRHYDPSSPLCQGPQNLTDVINQKHENIKYLPGITLPSNLHANPS 2177260

Query: 97      IKEAVKDASILVFNLPHQFIGKTLDQIKGHHLPYARGVSCIKGVDVSDGKVTLFSELIMQ 156
                + +AVKD++ILVFNLPHQFI KT +QIKG  LPYARG+SCIKGVDV++  + LFSE I +
Sbjct: 2177259 LVDAVKDSTILVFNLPHQFIIKTCEQIKGKILPYARGISCIKGVDVNEEGIHLFSETIGK 2177080

Query: 157     KLGIYCGALSGANIAPEVAAERLCETTIGYDPPPMD--LTSSDGSPEDNLPHVDGQRQKD 214
                 LGIYCGALSGANIA EVA E+  E++I YDPP MD    S + SP  +  ++     KD
Sbjct: 2177079 ILGIYCGALSGANIASEVALEKWSESSIAYDPPHMDSKAPSPNRSPSTSTENLVQFEHKD 2176900

Query: 215     LSS--TDIKLERVPQDYPHVNDELLEKMFERPYFLVHVIKDVAGVALGGALKNIVALAAG 272
                +S   + +KL+ +P DYP V+  +L+ +F RPYF + V+ DVAGV+LGGALKN+VALAAG
Sbjct: 2176899 VSGQLSRVKLQALPSDYPPVDHAVLKSLFHRPYFHIRVVSDVAGVSLGGALKNVVALAAG 2176720

Query: 273     FVAGKQWGENTKAAIIRQGVGEMIRFGRTWFPSSVNEKTFTEESAGIADLVASCYGGRNV 332
                +V G  WG+N KAAI+R G+ EM++FG  +F +++N +TFTEESAG+ADL+ SC GGRN
Sbjct: 2176719 WVDGMGWGDNAKAAIMRVGLLEMVKFGEKFFGATINTRTFTEESAGVADLITSCSGGRNF 2176540

Query: 333     RSATHAVEKGVSVAEIEKTEMNGQKLQGVSTAQTVYGFLEKHGKTDEFPLFCAVY 387
                R A  ++E+  S+ ++E+TE+NGQKLQG  TA  V  FL+K G  +EFPL  AVY
Sbjct: 2176539 RCAKLSIERKQSIEKVEETELNGQKLQGTLTAVEVNNFLKKQGLEEEFPLLTAVY 2176375


>scaffold_8
          Length = 1733983

 Score = 34.7 bits (78), Expect = 0.15
 Identities = 19/58 (32%), Positives = 31/58 (53%)
 Frame = -2

Query: 209   GQRQKDLSSTDIKLERVPQDYPHVNDELLEKMFERPYFLVHVIKDVAGVALGGALKNI 266
              G  Q+      I L  V   Y H N  LLEK+ +R +F+V +++D+ G  +   L+N+
Sbjct: 53925 GGHQRTSKLNAIVLAVVTWYYSHANSSLLEKLRDRDFFVVVLVEDLGGG*VEILLRNV 53752


  Database: C:\Program Files\Bioedit\database\an.fasta
    Posted date:  Jul 26, 2006  4:44 PM
  Number of letters in database: 37,196,396
  Number of sequences in database:  143

Lambda     K      H
   0.315    0.134    0.394
```

```
Gapped
Lambda     K      H
   0.267   0.0410    0.140


Matrix: BLOSUM62
Gap Penalties: Existence: 11, Extension: 1
Number of Hits to DB: 17,900,696
Number of Sequences: 143
Number of extensions: 266035
Number of successful extensions: 1238
Number of sequences better than  1.0: 3
Number of HSP's better than  1.0 without gapping: 12
Number of HSP's successfully gapped in prelim test: 3
Number of HSP's that attempted gapping in prelim test: 1041
Number of HSP's gapped (non-prelim): 560
length of query: 407
length of database: 12,398,798
effective HSP length: 104
effective length of query: 303
effective length of database: 12,383,926
effective search space: 3752329578
effective search space used: 3752329578
frameshift window, decay const: 40,  0.1
T: 13
A: 40
X1: 16 ( 7.3 bits)
X2: 38 (14.6 bits)
X3: 64 (24.7 bits)
S1: 41 (21.6 bits)
S2: 71 (32.0 bits)
```

## 3. Appendix III

Southern blots of *A. niger A8* and *A10* digested by *Ssp*I and hybridized with the probe 1 (figure 2) and 2 (figure 3) described in the chapter 4 paragraph "material and methods". In the figure a, the presence of a band at 5852 bp for the wild type and a unique band at 4379 bp for the mutants *A10* and *A8* confirm an unique insertion of *pyrG* at the right locus (figure 1). In the figure b, the presence of a band at 5852 bp for the wild type, the unique band at 1161 bp concerning *A. niger A8* and the unique band at 2671 concerning *A. niger A10* confirm the right insertion at the right locus of *pyrG* and differentiate *A. niger* A8, where *pyrG* is surrounded by repeats, from *A. niger A10*.

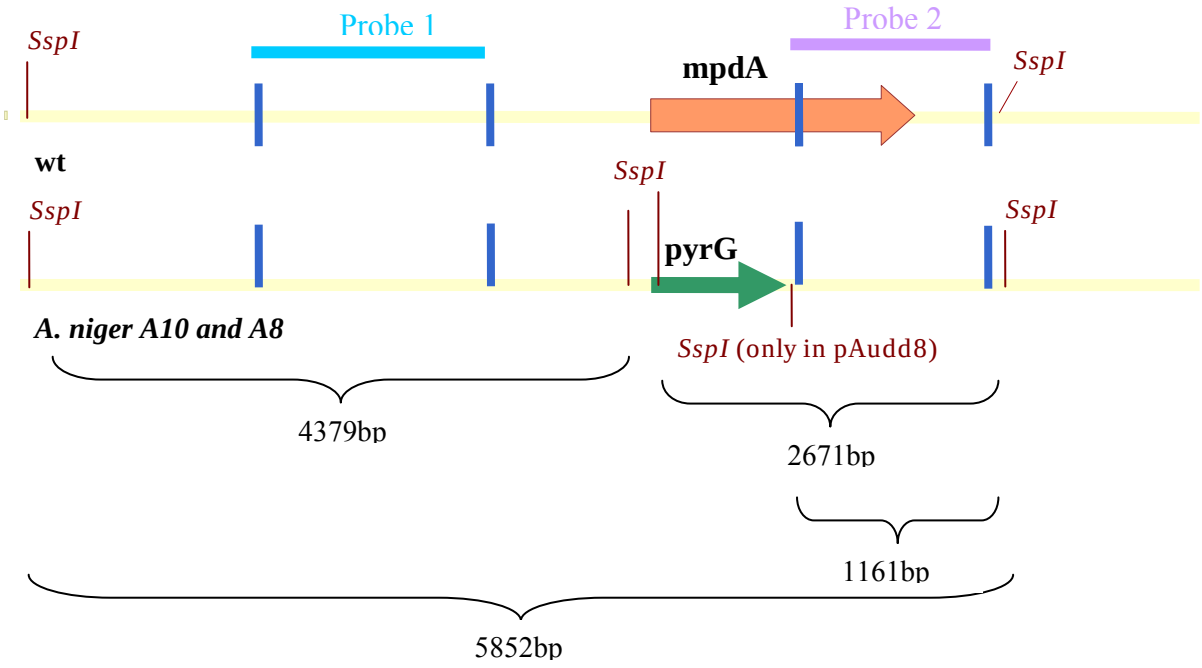



Figure 1: *mpdA* locus in *A. niger Jroy3*, *A8* and *A10*. Restriction sites of *Ssp*I, used for Southern blot analysis, are identified as well as the hybridation sites of the probes 1 and 2.

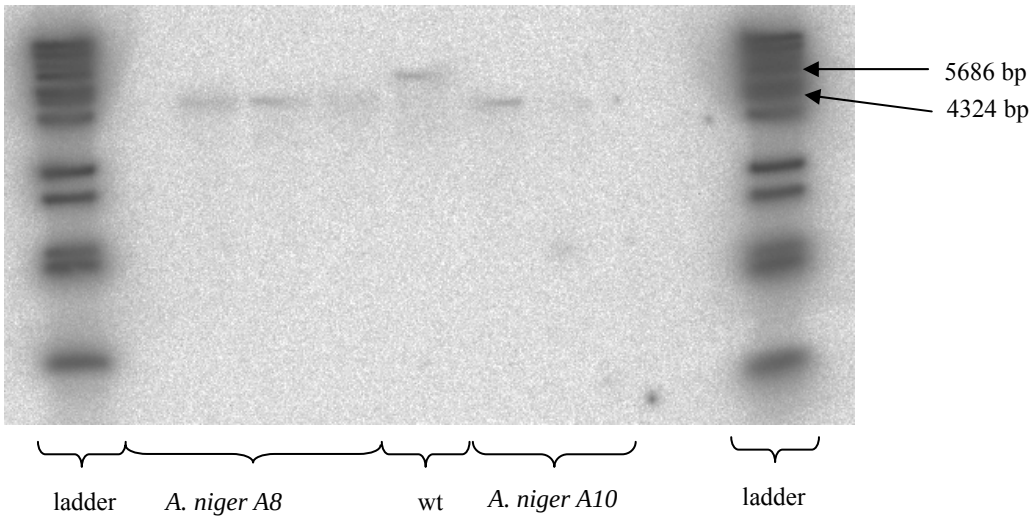



Figure 2: Southern blot of *A. niger A8* and *A10,* probe 1

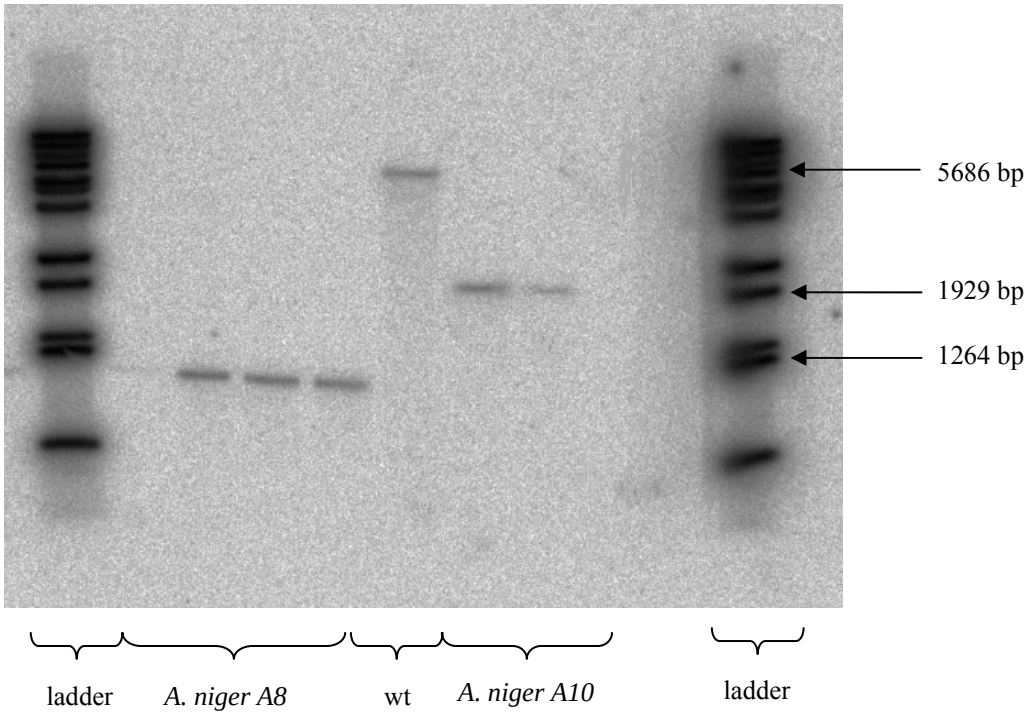
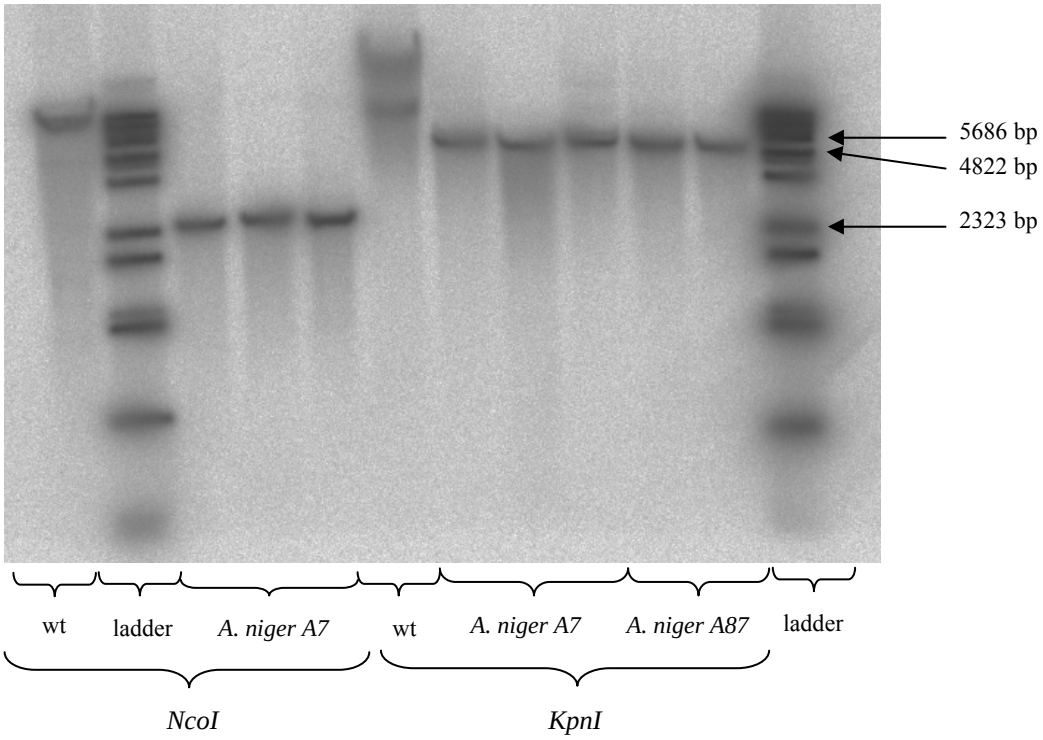



Figure 3: Southern blot of *A. niger A8* and *A10,* probe 2

## 4. Appendix IV

Southern blot of *A. niger A7* and *A87* digested by *Nco*I or KpnI and hybridized with the probe described in the chapter 4 paragraph "material and methods".

The probe used hybridizes downstream of *gfdA* gene. Moreover, the insertion of *pyrG* at the *gfdA* locus inserts a restriction site *Nco*I and a restriction site *KpnI*. For each mutant and each restriction enzyme, the unique band observed on the Southern blot, below the band of the wild type confirm an unique insertion of *pyrG* at the right locus.
Notes:
The two bands observed in the line 6 of the Southern, may be due to an incomplete digestion of DNA by *Kpn*I. This observation does not affect the conclusions of this experiment as the line 6 represents the wild type strain.
In the line 9, the presence of two bands, bigger than 5686bp, with very low intensity, can be observed. Therefore, this strain hasn't been used further.



## 5. Appendix V

Southern blots of *A. niger A87* digested by *Nco*I and hybridized with the probe 1 (figure 2), 2 (figure 3) and 3 (figure 4) described in the chapter 4 paragraph "material and methods".

The probe 1 hybridizes with the downstream area of *gfdA*. An insertion of *pyrG* at gfdA locus inserts a new *NcoI* restriction site. In the figure 2, the presence of a band at 8454bp for the wild type and the unique band at 2671 concerning *A. niger A87* confirm an unique insertion of *pyrG* at the right locus.

The hybridation locus of the probe 2 is shown figure 1. If *pyrG* has not been removed from *mpdA* locus, a band at 2564 bp should be seen as *pyrG* possesses a *NcoI* restriction site. In the figure c the presence of a unique band at 8454bp for the wild type and the mutants *A87* confirm the excision of *pyrG* at *mpdA* locus.

The hybridation locus of the probe 3 is shown figure 1. This probe 3 should hybridize on the DNA of the wild type but not on the DNA of the mutants. This is observed on the figure 4.

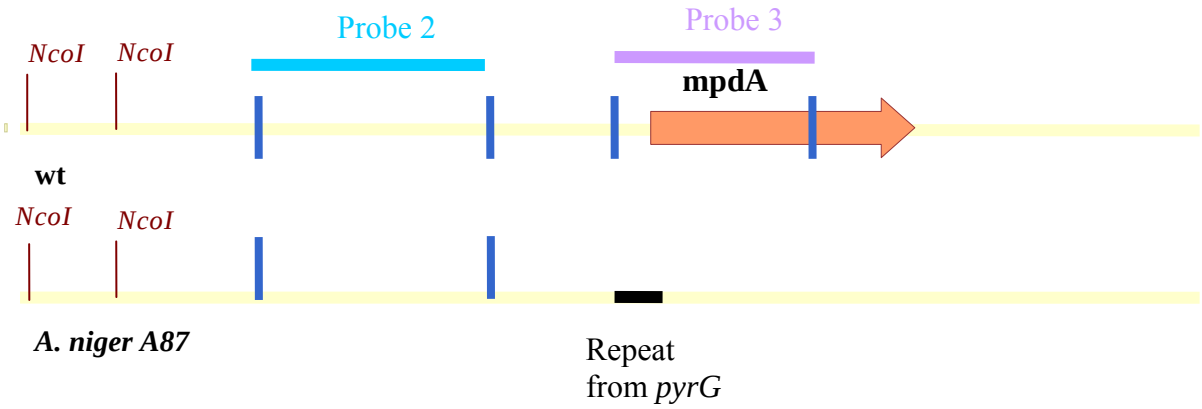



Figure 1: *mpdA* locus in *A. niger Jroy3* and *A87*. Restriction sites of *NcoI*, used for Southern blot analysis, are identified as well as the hybridation sites of the probes 2 and 3.

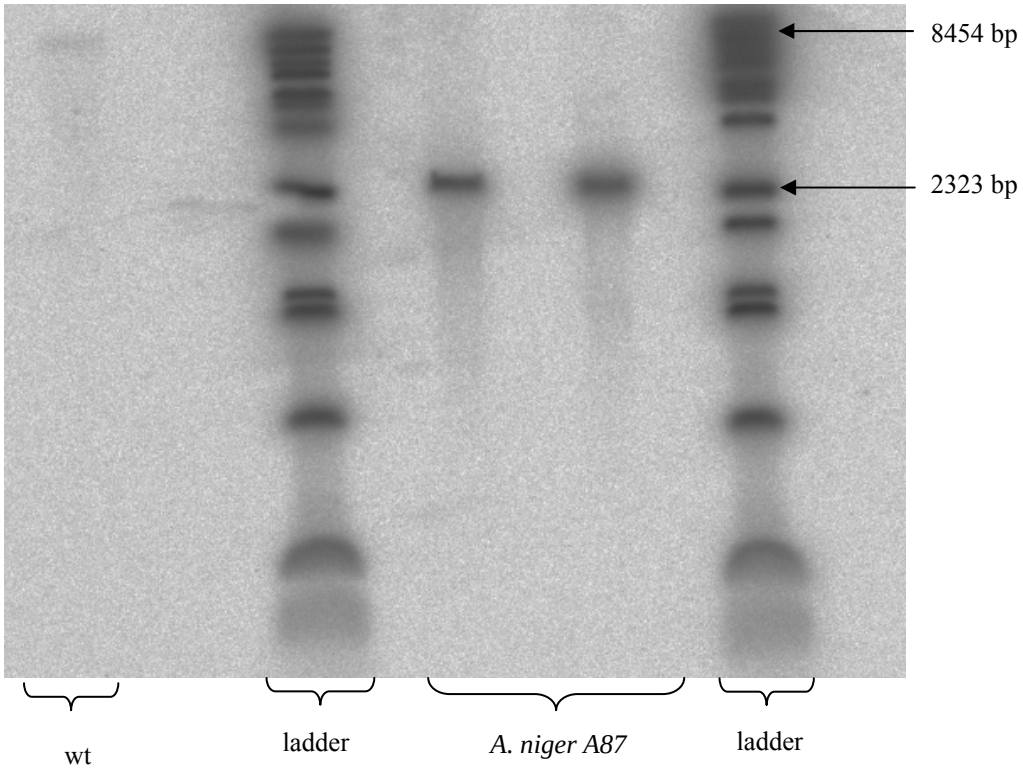



Figure 2: Southern blot of *A. niger A87,* probe 1

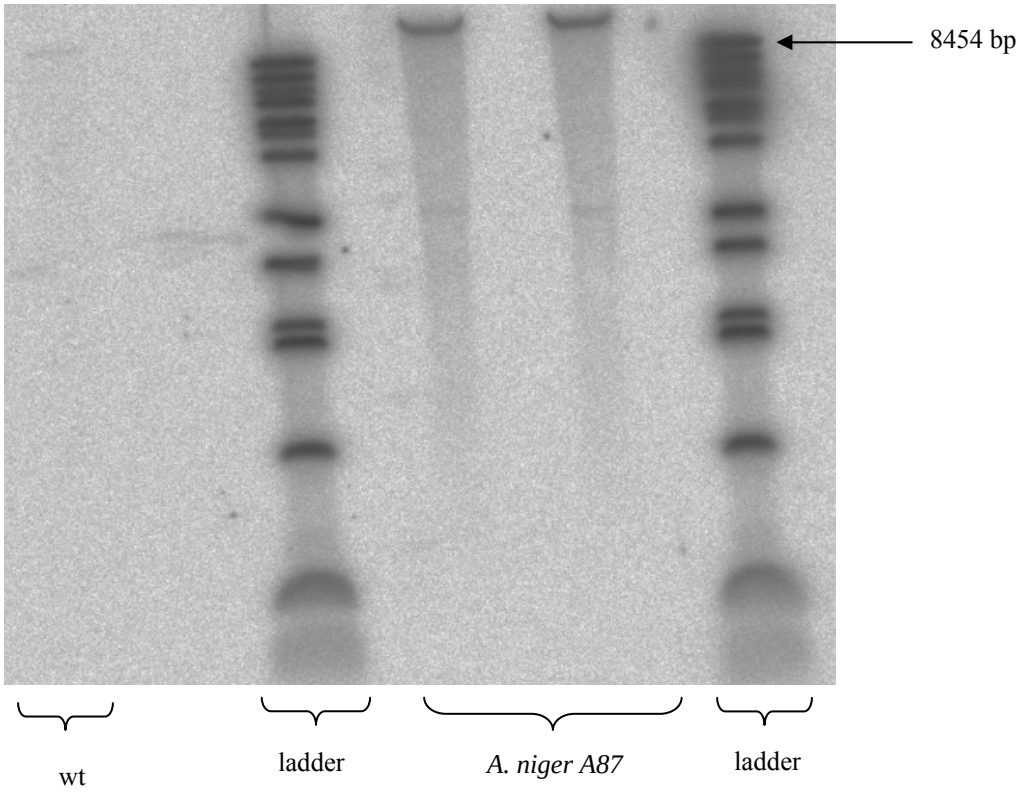



Figure 3: Southern blot of *A. niger A87,* probe 2

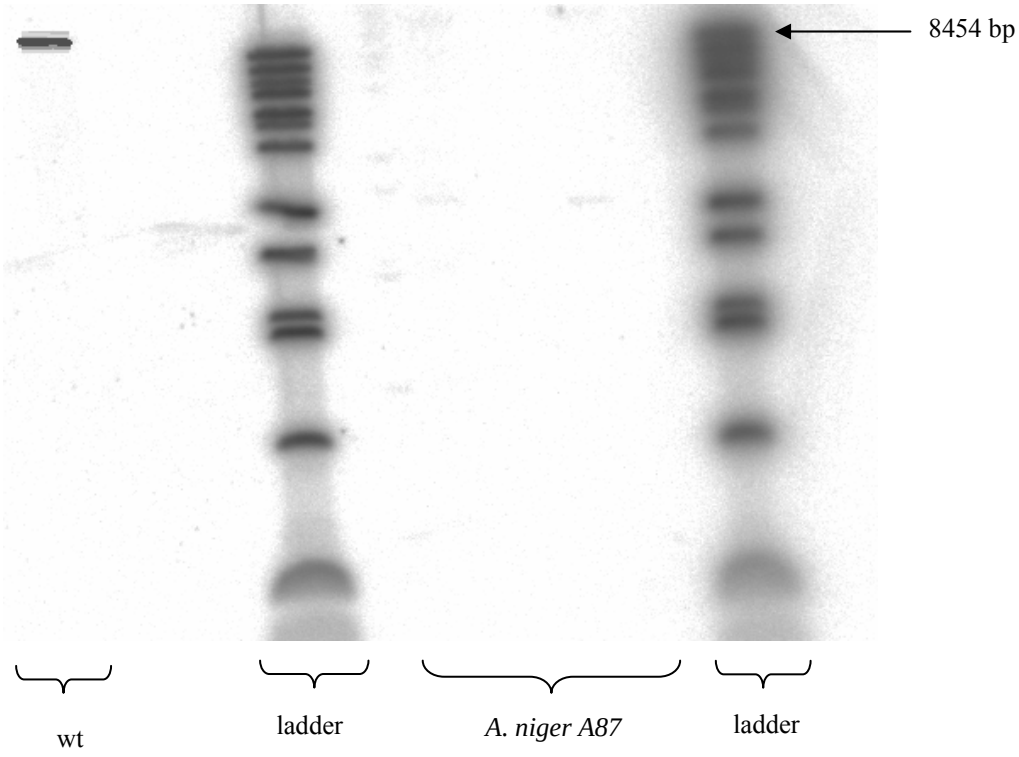
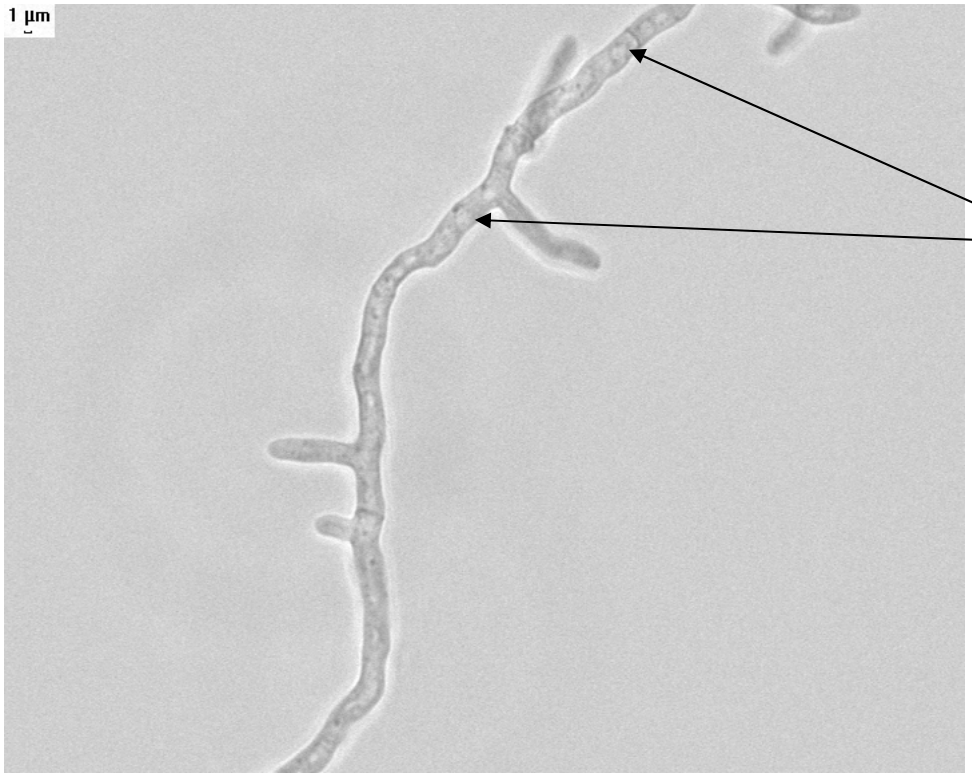



Figure 4: Southern blot of *A. niger A87,* probe 3

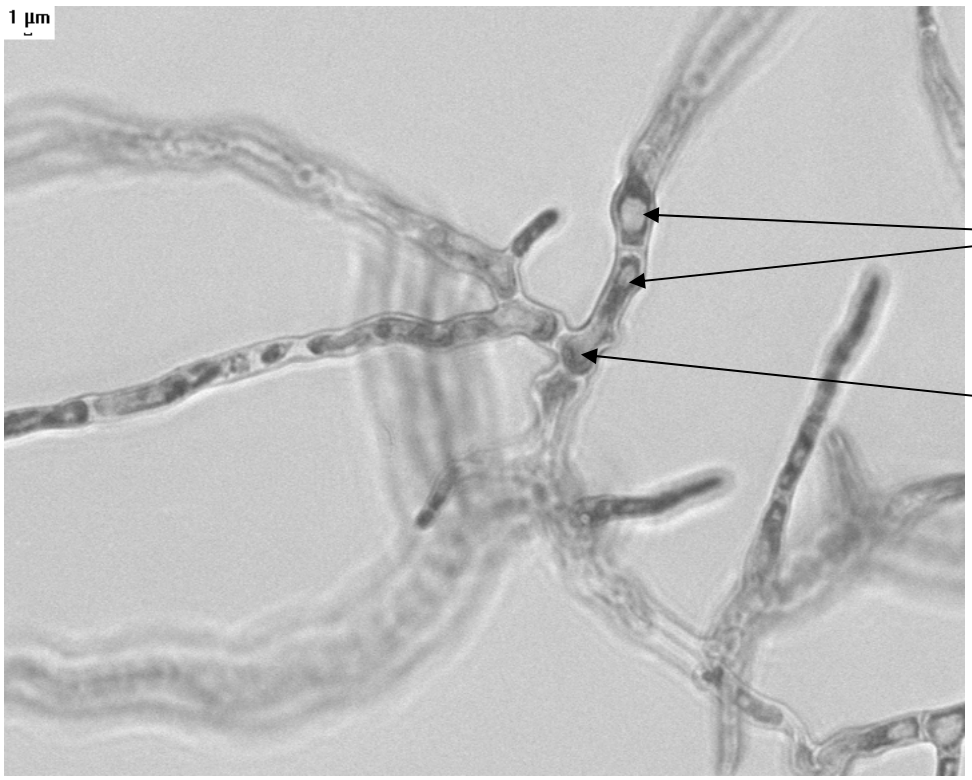
## 6. Appendix VI

Morpholgy of the wild type and mutants (*mpdA*, *gfdA* and double deletion *mpdA/gfdA*) in exponential phase (pictures 1) and oxygen limitation phase (pictures 2).

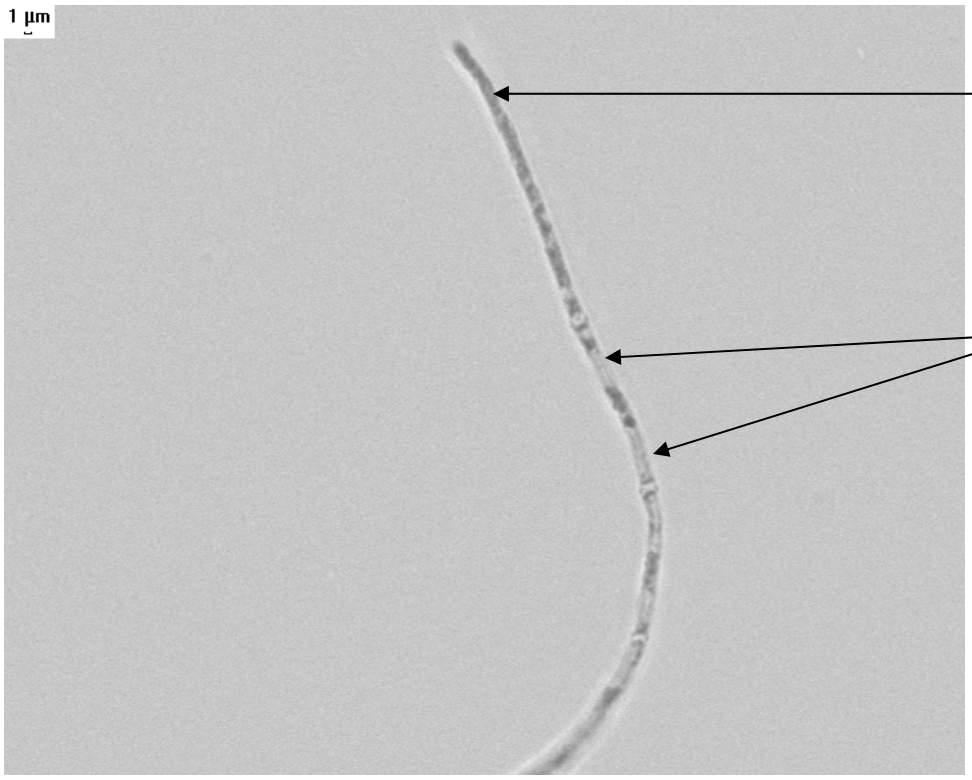



Wt-1

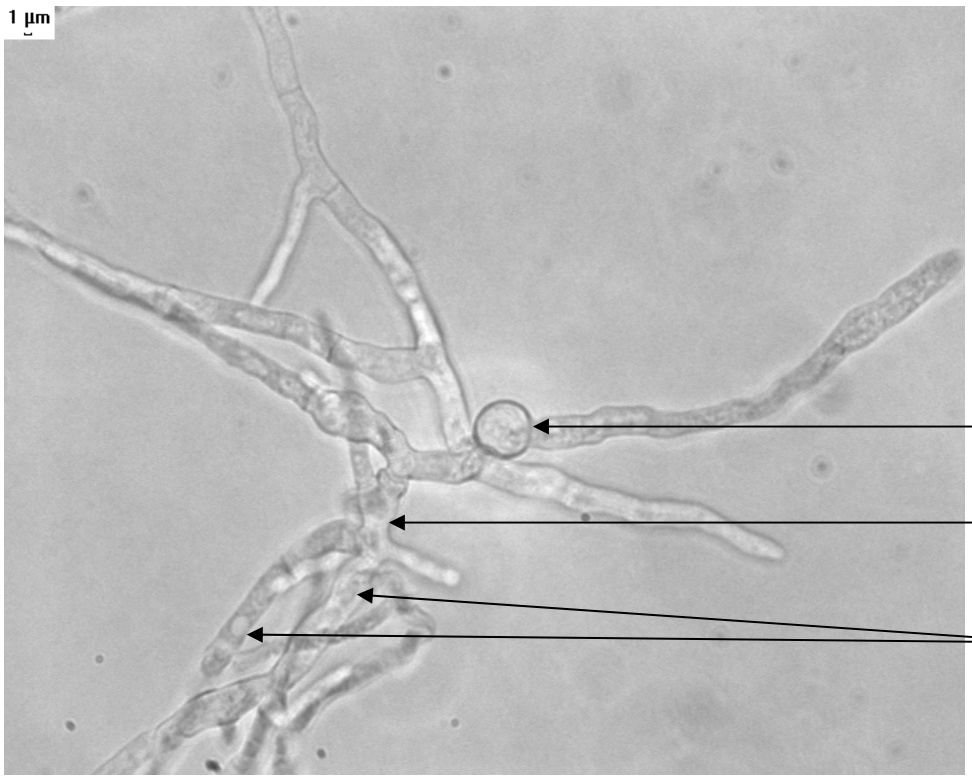

Wt-2


1 µm
Ovale vacuoles
Swollen hyphae in the vicinity of branching areas

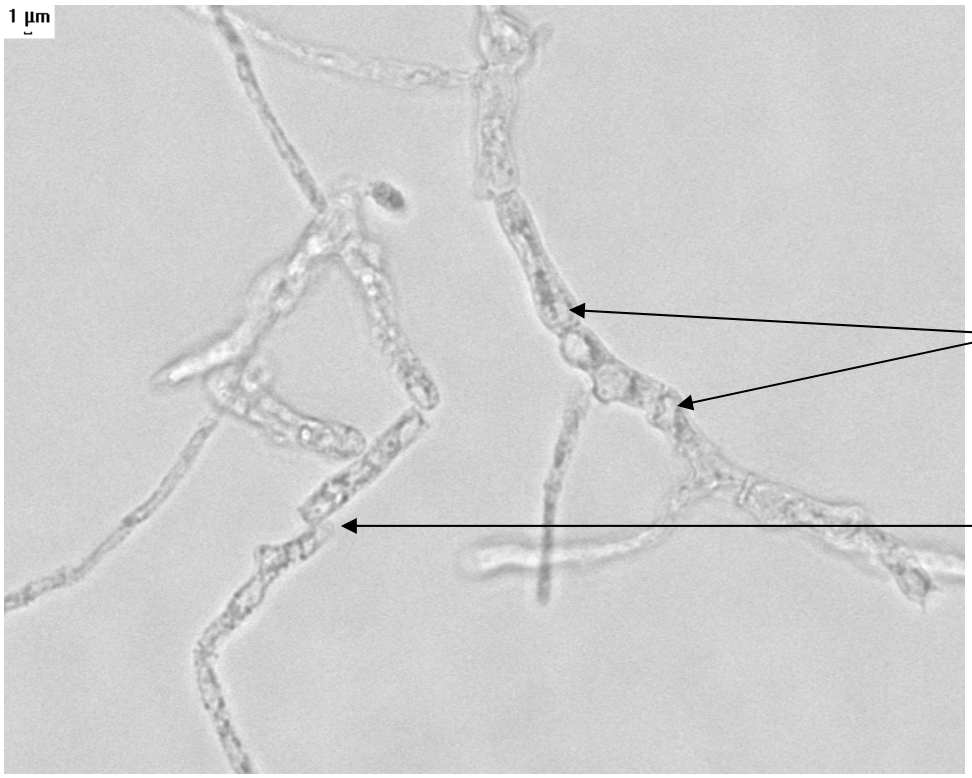
mpdA-1


1 µm
Very thin tip
Long vacuoles

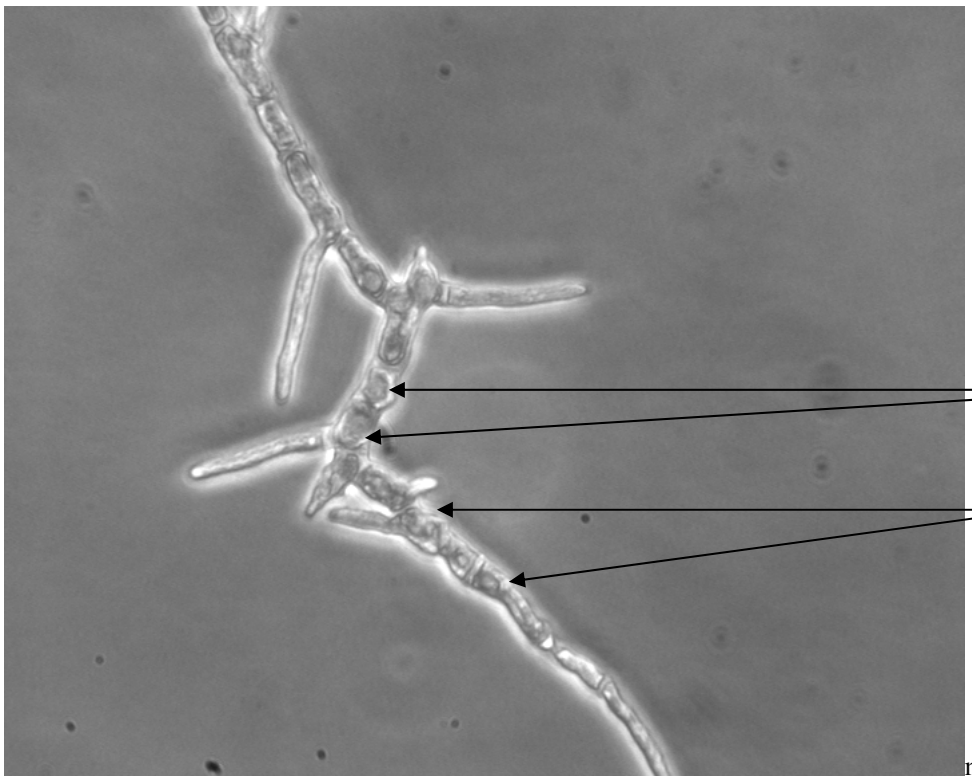
mpdA-2


1 µm
Balloon-like structure
Irregular hyphae
Vacuoles

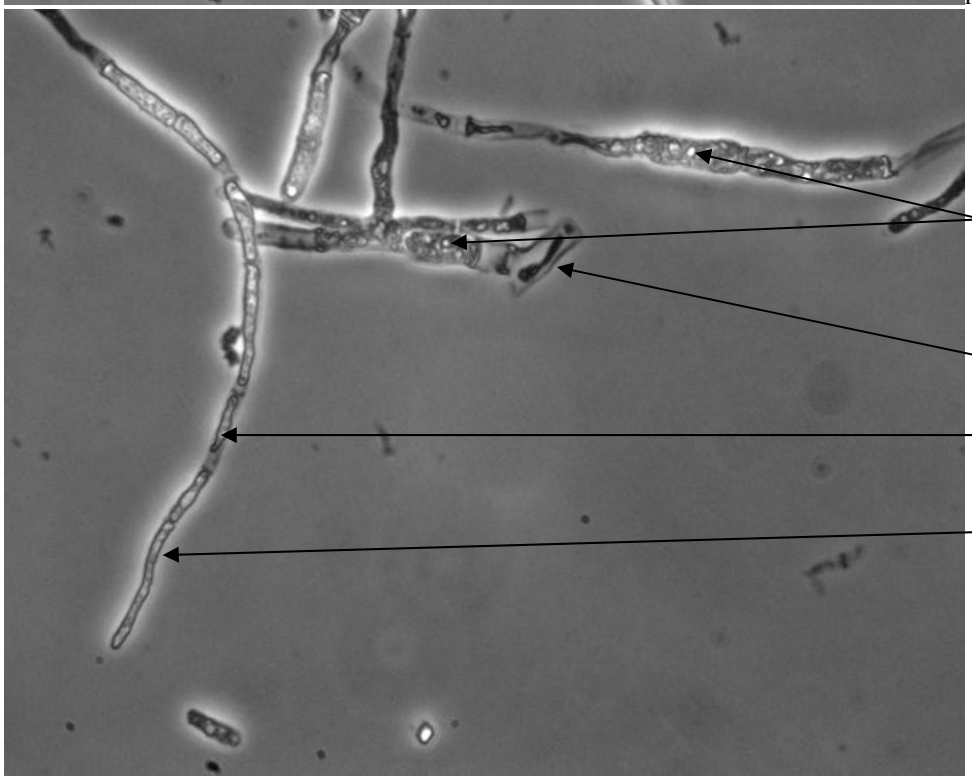
gfdA-1


1 µm
Small vacuoles
Fragmented hyphae

gfdA-2




mpdAgfdA-1




mpdAgfdA-2